University of Alberta

Distribution of sortilin in the adult rat brain and its association with the p75 neurotrophin receptor

By

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ABSTRACT

Neurotensin receptor-3/sortilin is a single trans-membrane domain protein involved in intracellular trafficking/sorting of lysosomal enzymes. Recent evidence indicates that p75 neurotrophin receptor ($p75^{NTR}$) interacts with sortilin to mediate cell death induced by pro-neurotrophins. In the present study, I evaluated normal distribution of sortilin in the adult rat brain and its possible alterations following 192 IgG-saporin treatment. Using Western blotting and immunohistochemical methods, I demonstrated that sortilin is widely distributed in the normal adult rat brain. A subset of sortilin-immunoreactive neurons was found to express $p75^{NTR}$ immunoreactivity. Additionally, sortilin immunoreactivity was evident in cholinergic neurons of the basal forebrain, striatum and brainstem region. Administration of 192 IgG-saporin induced a significant a loss of $p75^{NTR}$ positive cholinergic neurons, but no alteration in the levels/expression of sortilin, in the treated rats. These results suggest that although sortilin is distributed widely in the adult rat brain and is co-localized with $p75^{NTR}$ positive cells, its levels/expression may be differentially regulated than $p75^{NTR}$ levels/expression.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ANOVA	Analysis of variances
BDNF	Brain derived neurotrophic factor
ChAT	Choline acetyltransferase
CNS	Central nervous system
DAB	Diaminobenzidine tetrahydrochloride
DBB	Diagonal band of Broca
ECL	Enhanced chemiluminescence
FAP-1	Fas-associated phosphatase-1
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
GGA	Golgi associated, γ-adaptin homologous, ARF binding protein
GM2AP	GM2 ganglioside activator protein
GST	glutathione S-transferase fusion protein
icv	intracerebroventricular
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LpL	Lipoprotein lipase L
MAGE	Melanoma antigen
MAP	Mitogen-activated protein
MCID	Microcomputer Imaging Device
MCP-1	Monocyte chemotactic protein 1
MIP2	Macrophage inflammatory protein-2
NADE	$p75^{NTR}$-associated cell death executor
NBM	Nucleus basalis of Meynert
NGF	Nerve growth factor
NRAGE	Neurotrophin receptor-interacting MAGE homologue
NRIF	Neurotrophin receptor interacting protein
NT3	Neurotrophin-3

NT4	Neurotrophin-4
NTR1	Neurotensin receptor-1
NTR2	Neurotensin receptor-2
NTR3	Neurotensin receptor-3
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffer saline
PC1	Pro-convertase 1
PC2	Pro-convertase 2
Pro-BDNF	Pro-Brain derived neurotrophic factor
Pro-NGF	Pro-Nerve growth factor
RAP	Receptor-associated protein
RIP-2	Receptor interacting protein-2
ROD	Relative optical density
RT-PCR	Reverse transcription-polymerase chain reaction
SAP	Sphingolipid activator protein
SC-1	Schwann cell factor-1
siRNAi	small interfering RNA interference
SorCS	Sortilin-related Vps10 domain-containing receptor
SorLA	Sortilin-related receptor containing LDLR class A repeats
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TrkA	Tropomyosin-related tyrosine kinase A receptor
TrkB	Tropomyosin-related tyrosine kinase B receptor
TrkC	Tropomyosin-related tyrosine kinase C receptor
VAChT	Vesicular acetylcholine transporter

CHAPTER 1
INTRODUCTION

INTRODUCTION

1. Neurotrophins and their receptors

1.1. Neurotrophins

The neurotrophins comprise a small family of secreted proteins that promote the growth, survival, and differentiation of neurons in the central and peripheral nervous systems. Constant exposure to neurotrophins is an absolute requirement for the survival and development of neurons (Mamidipudi and Wooten, 2002). In mammals, the known neurotrophins are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) (Bibel and Barde, 2000; Huang and Reichardt, 2001; Kalb, 2005). These proteins share many functional properties with classical neurotransmitters. For example, they are released at synapses and are required to be activated for activity-dependent forms of synaptic plasticity. Neurotrophins can also affect membrane permeability for sodium ions on a similar time-scale as neurotransmitters (Kafitz et al., 1999; Thoenen, 2000; Poo, 2001; Blum and Konnerth, 2004). In addition to their biological roles, neurotrophins have been implicated in several neurological disorders, such as Alzheimer's disease (AD) (Burbarch et al., 2004; Lee et al., 2005), Parkinson's disease (Hyman et al., 1991, Porritt et al., 2005), and epilepsy (Tongiorgi et al., 2004).

Like several peptide neuromodulators, neurotrophins are synthesized as precursor forms (pro-neurotrophins) and proteolytically processed to generate the mature neurotrophin. Mammalian neurotrophin genes encode for glycosylated precursors (31-35kDa), which are subsequently cleaved intracellularly by proteolytic enzymes, such as furin and pro-convertases 1 and 2 (PC1 and PC2), to release the mature neurotrophins (see Figure 1.1; Roux and Barker, 2002). Neurotrophins are secreted both constitutively and by regulated pathways in neurons and non-endocrine cells. Mature neurotrophins have been shown to be involved in a variety of functions, including neuronal survival and maintenance of normal and activity-dependent functions of the nervous system (Seidah et al., 1996; Mowla et al., 1999; Mamidipudi and Wooten, 2002). Recent findings indicate that

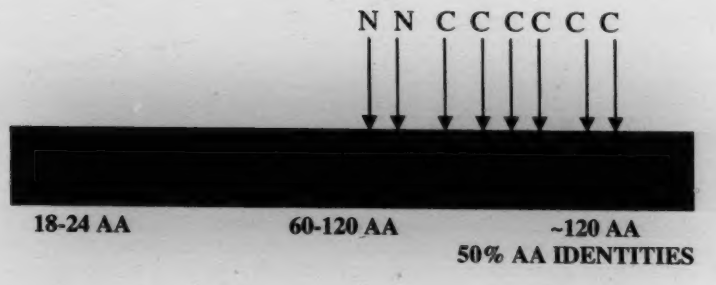


Figure 1.1. Schematic representation of the cleavage of neurotrophin precursors (pro-neurotrophins) by proteolytic enzymes. The biologically active carboxy-terminal (depicted in green) is about 50% identical in all 4 neurotrophins. The mature protein (depicted in green) includes a characteristic pattern of 6 cysteine residues (C) that form three conserved cysteine bridges. The amino-terminal half of the proteins is functional in the biosynthesis of the proteins and includes a signal peptide (depicted in red) which mediates membrane transport and a pro-peptide (depicted in purple) which is important for protein folding. S.S, signal sequence; *N*, N-glycosylation sites.

pro-neurotrophins such as pro-NGF and pro-BDNF are also secreted and have biological activity in the normal brain (Lee et al., 2001; Dechant and Barde, 2002; Barker, 2004).

1.2. Neurotrophin receptors

The broad spectrum of biological activities exerted by the mature neurotrophins result from their ability to bind and activate two types of structurally unrelated receptors, the p75 neurotrophin receptor ($p75^{NTR}$) and the three members of the tropomyosin-related tyrosine kinase (Trk) receptor family i.e., TrkA, TrkB and TrkC. While $p75^{NTR}$ binds all neurotrophins with similar affinities, TrkA preferentially interacts with NGF, TrkB with BDNF and NT4, and TrkC with NT3 (Kaplan and Miller, 1997; Friedman and Greene, 1999; Patapoutian and Reichardt, 2001; Dechant and Barde, 2002) (Figure 1.2). Bioactive mature neurotrophins, which are homodimers, can simultaneously engage two Trk receptors in a high-affinity complex that mediates receptor auto-phosphorylation and activation of well defined signaling pathways. Ultimately, the Trk receptor stimulates neuronal survival, differentiation, neurite outgrowth and myelination, in addition to synaptic plasticity and function (Huang and Reichardt, 2003; Nykjaer et al., 2005).

1.3. The structure of $p75^{NTR}$

$p75^{NTR}$ was the first-discovered member of the tumour necrosis factor (TNF) receptor superfamily, which mediates cellular differentiation and apoptosis (Chao, 1994). The human $p75^{NTR}$ is synthesized as a soluble precursor containing a 28-amino acid signal peptide, which is cleaved from the molecule upon membrane insertion of the receptor. This leaves behind a 399 amino acid transmembrane protein that is *N*-glycosylated in the extracellular domain and *O*-glycosylated in the juxtamembrane or "stalk" domain (Large et al., 1989; Roux and Barker, 2002; Schor, 2005). Structurally, the $p75^{NTR}$ receptor is divided into three parts: an extracellular neurotrophin-binding domain, a transmembrane domain and an intracellular domain. The extracellular domain contains a module of cysteine

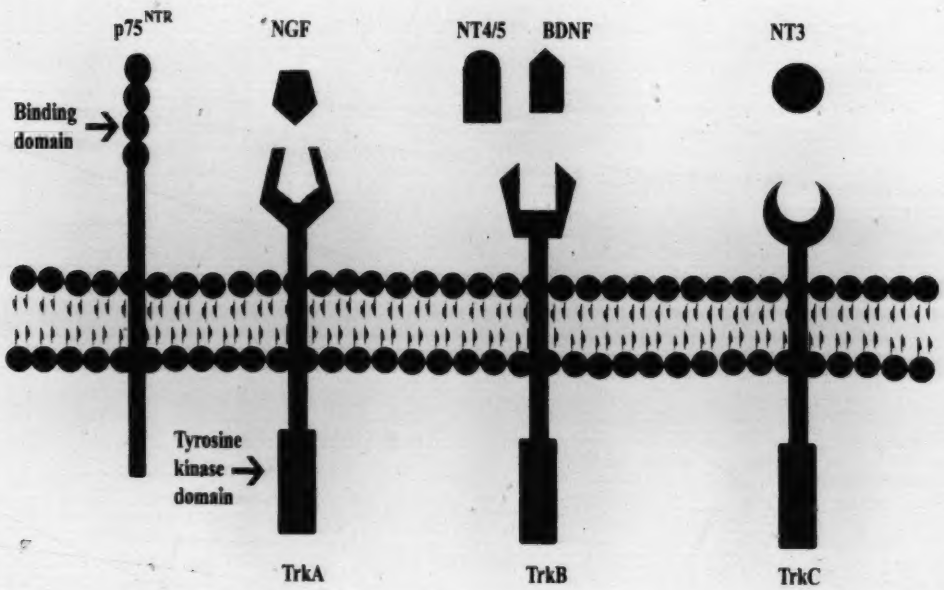


Figure 1.2. Diagram showing the interaction and differing affinities of neurotrophin receptors, $p75^{NTR}$ and Trks, for neurotrophins. The low-affinity neurotrophin receptor p75 binds all of the neurotrophins with similar affinity. However, neurotrophin family members bind preferentially to various Trk receptors. TrkA preferentially interacts with NGF, TrkB with BDNF and NT4, and TrkC with NT3. *NT,* neurotrophin; *BDNF,* brain-derived neurotrophic factor, *NGF,* nerve growth factor.

residues which is a characteristic feature of the TNF receptor superfamily. The transmembrane domain contains a γ-secretase cleavage site, whereas the intracellular cytoplasmic tail contains a "death domain" of about 80 amino acids as reported in a sub-group of "death receptors" associated with the TNF receptor superfamily. A notable difference is that while other members of the TNF receptor superfamily bind to trimeric ligands, two monomeric molecules of $p75^{NTR}$ are required to bind a homodimer of NGF (Roux and Barker, 2002; Barker, 2004; He and Garcia, 2004; Schor, 2005).

1.4. Expression of $p75^{NTR}$ in the central nervous system (CNS) and its localization in cholinergic neurons

$p75^{NTR}$ mRNA and protein are highly expressed in many cell populations of the CNS during mammalian development (Roux and Barker, 2002). However, it is down-regulated in the adult brain where it is found to be expressed only in selected neuronal populations. Nonetheless, $p75^{NTR}$ re-expression occurs under certain experimental conditions such as mechanical damage, focal ischemia, axotomy, as well as in stroke, thus raising the possibility of a role for this receptor in the degeneration of neurons (Dechant and Barde, 2002).

In the normal adult rat brain, $p75^{NTR}$ is expressed primarily in the basal forebrain cholinergic neurons and Purkinje cells of the cerebellum. The cholinergic neurons in the brain are usually evident in two basic organizations: local interneurons and projection neurons. Interneurons expressing cholinergic phenotype are located primarily in the striatum, olfactory tubercle and the islands of Calleja. As for projection neurons, there are two major groups: i) the basal forebrain cholinergic neurons, which include the medial septum, diagonal band of Broca (DBB), substantia innominata and nucleus basalis of Meynert (NBM), provide innervation to the entire cortex and hippocampus, and ii) the pontomesencephalotegmental cholinergic neurons which provide projection to the diencephalon, brainstem and cerebellum (Figure 1.3) (Cooper et al., 1996; Mufson et al., 2003). Although most

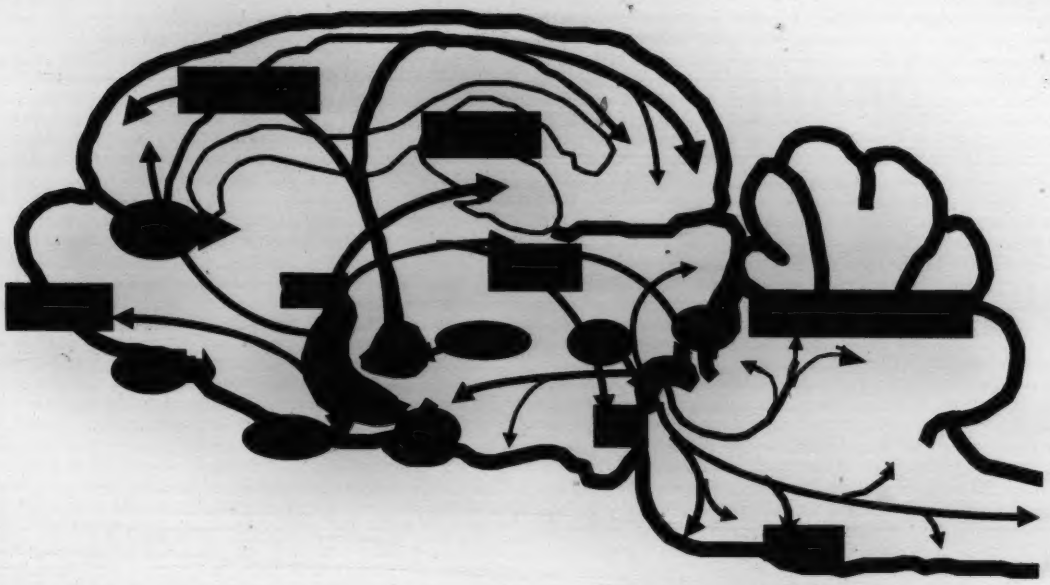


Figure 1.3. Anatomy of the central cholinergic system and its projections in the adult rat brain. Central cholinergic neurons are organized into two basic groups, i) local circuit cells, and ii) projection neurons. Local circuit neurons include interneurons within the striatum (STR), nucleus accumbens, olfactory tubercle (OLFB) and Islands of Calleja complex (ICJ), including cells of the medial septum (MS), diagonal band of Broca (DBN), substantia innominata (SI), magnocellular preoptic field and nucleus basalis (BAS); and b) the pontomesencephalotegemental cholinergic complex, which is comprised of cells in the pendunculopontine (PP) and laterodorsal tegmental nuclei (TN). The latter group of cells has ascending projections to the thalamus (THA) and descending projections to the pontine (PRN) and medullary reticular formations, deep cerebellar (CERE NUCLEI) and vesibular nuclei and cranial nerve nuclei. HIPPO, Hippocampus; IP, interpeduncular nucleus.

of the cholinergic neurons located in the basal forebrain region express $p75^{NTR}$, a subset of cholinergic neurons located in the ventral palladium and ventral to the lenticular nucleus are found to be $p75^{NTR}$-negative. Other cholinergic neurons located in the striatum, olfactory tubercle or in the upper brainstem regions are also found not to express $p75^{NTR}$. In the cerebellum, the Purkinje cells, which are non-cholinergic, have been shown to express $p75^{NTR}$ (Heckers et al., 1994; Bussmann and Sofroniew, 1999; Ferreira et al., 2001; Greferath et al., 2002).

Assimilated evidence suggests that preferential loss of the basal forebrain cholinergic neurons contributes to the progressive cognitive deficits observed in AD pathology. However, at present, the underlying cause of degeneration of these neurons remains unclear. The immunotoxin 192 IgG-saporin, a ribosomal-inactivating toxin coupled to a monoclonal antibody against the rat $p75^{NTR}$, which is taken up selectively by forebrain cholinergic neurons expressing $p75^{NTR}$, has been used extensively to model the cognitive and neurochemical sequelae of cholinergic hypofunction observed in AD brains. Other non-cholinergic cell groups of the basal forebrain and the $p75^{NTR}$-negative cholinergic neurons of the striatum and basal forebrain regions remain unaffected by the toxin. At present, however, the underlying mechanisms by which 192 IgG-saporin triggers the loss of the basal cholinergic neurons have yet to be established (Heckers et al., 1994; Rossner, 1997; Perry et al., 2001, Hawkes et al., 2005). Studies using receptor autoradiography and *in situ* hybridization of receptor mRNA in the AD brain have shown a decrease in neurotrophin receptor levels, possibly implicating neurotrophin decline as one of the underlying causes of the loss of basal forebrain neurons in AD (Strada et al., 1992; Boissiere et al., 1997). A study by Yeo and colleagues used $p75^{NTR}$-/- mice to observe that although there was no effect on adult striatal cholinergic neurons, the absence of $p75^{NTR}$ resulted in hypertrophy of Trk-A-immunoreactive neurons (Yeo et al., 1997).

1.5. p75^{NTR} interactors

The p75^{NTR} is a complex receptor possessing a myriad of cellular interactors, such as neurotrophin receptor-interacting MAGE homologue (NRAGE) [MAGE for melanoma antigen], TNF receptor-associated factors (TRAFs), Trks, neurotrophin receptor interacting protein (NRIF), p75^{NTR}-associated cell death executor (NADE), Schwann cell factor-1 (SC-1), receptor interacting protein (RIP-2), Fas-associated phosphatase-1 (FAP-1), as well as caveolin-1 (Casademunt et al., 1999; Chittka and Chao, 1999; Irie et al., 1999; Mukai et al., 2000; Salehi et al., 2000; Schor, 2005). By interacting with these molecules, p75^{NTR} acts as a multi-functional receptor that can mediate various cellular functions (Schor, 2005).

1.6. Functions of the p75^{NTR}

The p75^{NTR} is known to be involved in a wide array of cellular processes, including neuronal migration, regulation of axonal outgrowth and neuronal survival as well as modulation of neuronal phenotype during development (Dechant and Barde, 2002; Schor, 2005). Paradoxically, the p75^{NTR} has been shown to exhibit both pro- and anti-apoptotic effects. It can act as an accessory to the Trk receptor by interacting with its cytosolic and transmembrane domains, thereby stimulating or hampering Trk signaling. In doing so, p75^{NTR} assumes the role of a co-receptor that refines Trk affinity and specificity for neurotrophins (Chao and Hempstead, 1995; Huang and Reichardt, 2003; Epa et al., 2004; Hannila et al., 2004). This is supported, in part, by the evidence that p75^{NTR} can be co-immunoprecipitated with Trks A, B and C in A293 cells transfected with both receptor types (Bibel et al., 1999). In addition to modulating Trk-mediated functions, p75^{NTR} also controls and conveys Trk-independent activities (Chao, 2003; Nykjaer et al., 2005).

A well established function of p75^{NTR} is to promote cell death in the nervous system in both *in vivo* and *in vitro* paradigms (Dechant and Barde, 2002; Barker, 2004). Like other members of the TNF receptor superfamily, the p75^{NTR} possesses an intracellular "death domain" which is capable of mediating

programmed cell death upon ligand binding. It has also been suggested that in some developing neurons, $p75^{NTR}$ can trigger a constitutive death signal, which is silenced in the presence of Trk receptors, thereby mediating survival (Miller and Kaplan, 2001). Recent studies indicate that complete deletion of the $p75^{NTR}$ leads to an increase in the number of forebrain cholinergic neurons, thus suggesting a potential role for the $p75^{NTR}$ in the degeneration of these neurons (Naumann et al., 2002; Dechant and Barde, 2002; Nykjaer et al., 2005). Additionally, apoptosis of cultured neuronal cell lines, glia and a variety of primary neurons is known to be mediated by activation of $p75^{NTR}$ (Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Davey and Davies, 1998; Soilu-Hanninen et al., 1999).

Despite the wealth of research on $p75^{NTR}$, the precise ligand it requires to induce apoptotic effects under *in vitro* or *in vivo* conditions remains unclear. Mature neurotrophins are not effective activators of $p75^{NTR}$-induced apoptosis, and high non-physiological concentrations are often required to induce even modest levels of cell death (Barker, 2004; Nykjaer et al., 2005). Additionally, there is mounting evidence to indicate that NGF and pro-NGF have opposing effects on cell survival and cell death, respectively (Nykjaer et al., 2004). Using a furin-resistant form of pro-NGF, it was shown that unprocessed NGF binds $p75^{NTR}$ with high-affinity and is a potent inducer of $p75^{NTR}$-dependent apoptosis in sympathetic neurons, oligodendrocytes and in a vascular smooth muscle cell line (Lee et al., 2001; Nykjaer et al., 2004). The ability of pro-NGF to activate $p75^{NTR}$ over TrkA was initially believed to reflect differences in affinity for the two receptors. However, pro-NGF binds equally well to $p75^{NTR}$ and TrkA, thus raising questions as to the underlying mechanisms mediating the effects of pro-NGF. Recent studies provide compelling new evidence that pro-NGF induces cell death by interacting simultaneously with $p75^{NTR}$ as well as sortilin – a receptor that has been characterized to bind the neuropeptide neurotensin (Nykjaer et al., 2004) (Figure 1.4).

2. Neurotensin and its receptors

2.1.Neurotensin

Neurotensin is a tri-decapeptide isolated originally from the bovine hypothalamus by Carraway and Leeman in 1973. It acts as a neuromodulator in the CNS and as a tissue hormone in the periphery. Centrally, neurotensin has been implicated in multitude of functions, including nociception, thermoregulation, neuroendocrine regulation and dopamine transmission in nigrostriatal and mesolimbic pathways. In the periphery, neurotensin has been shown to regulate blood pressure, gastric acid secretion and lipid digestion (Nemeroff et al., 1992; Rosténe and Alexander, 1997; Sarret and Beaudet, 2003).

2.2. Neurotensin receptors

The actions of neurotensin in the CNS and peripheral tissues are known to be mediated by three transmembrane neurotensin receptors (NTR) referred to as NTR1, NTR2 and NTR3/sortilin (Vincent et al., 1999). Pharmacological identification of the first two receptors, i.e. NTR1 and NTR2, shows that they are traditional seven-transmembrane G-protein-coupled receptors which are distributed widely throughout the CNS and are known to modulate a variety of neurotensin functions. The NTR3 receptor, unlike NTR1 and NTR2, is a single transmembrane receptor identical to the sorting protein, sortilin, isolated originally from human brain homogenates (Tanaka et al, 1990; Vita et al, 1993; Mazella et al., 1996; Petersen et al., 1997; Mazella et al., 1998). NTR1 is a high-affinity neurotensin receptor whose intracellular signaling pathway is well established and known to involve phosphatidylinositol hydrolysis, intracellular calcium release as well as activation of mitogen-activated protein (MAP) kinase (Chabry et al., 1994; Poinot-Chazel et al., 1996). SR48692 is a selective non-peptide antagonist on this receptor that blocks all effects of neurotensin while SR142948A is a non-selective antagonist (Gully et al., 1993; Gully et al., 1997). The signaling of the levocabastine-sensitive NTR2 is more controversial and varies with the species from which the receptor is isolated, as well as the cell system used (Mazella et al., 2001). Interestingly, SR48692 and SR142948A,

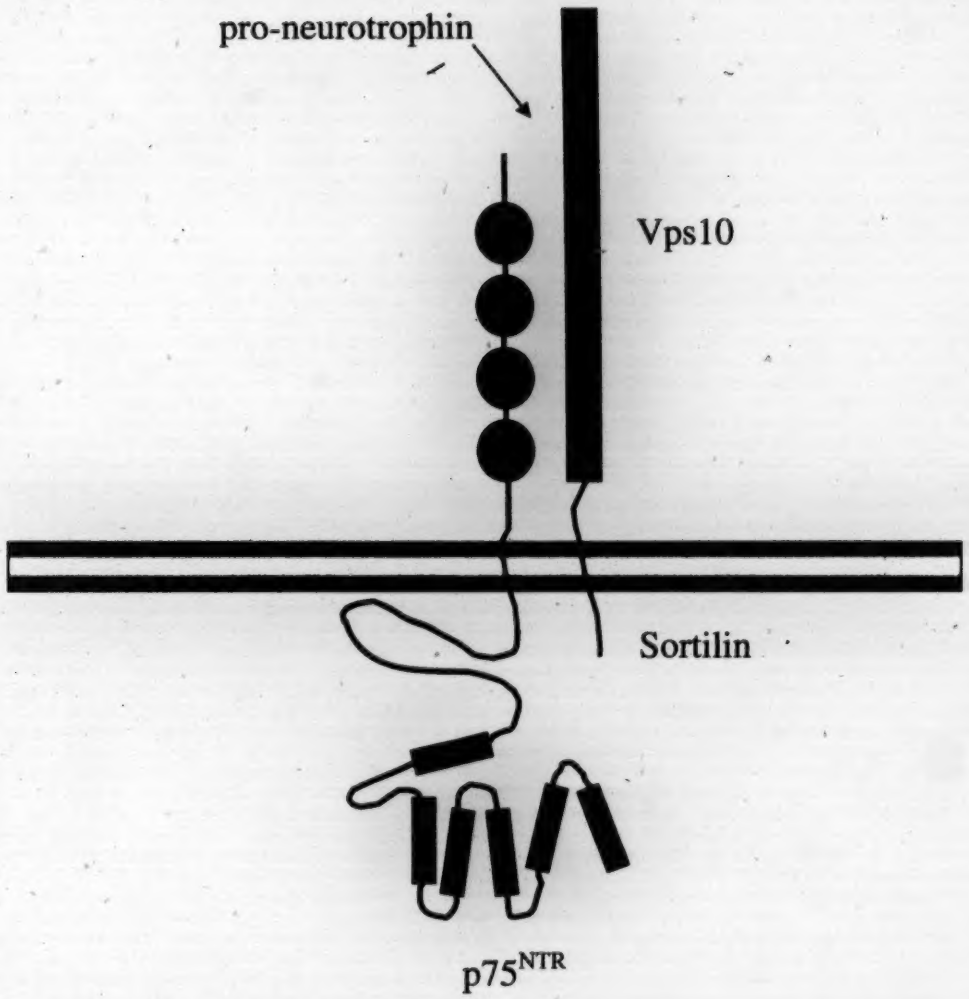


Figure 1.4. Interaction of sortilin and $p75^{NTR}$ to form a receptor complex at the cell surface which can mediate apoptotic cell death induced by pro-neurotrophins.

although antagonists on the NTR1 receptor, have agonist actions on the NTR2 receptor (Botto et al., 1997; Yamada et al., 1998).

2.3. Neurotensin receptor-3/Sortilin

Petersen et al. (1997) used receptor-associated protein (RAP) affinity chromatography to purify and characterize a novel 100kDa glycoprotein from membrane protein extracts of the human brain. Further characterization of this glycoprotein indicated that gp95/sortilin is synthesized as a precursor pro-peptide, which is incapable of ligand binding. The precursor peptide is usually converted to the mature ligand-binding receptor by furin-mediated cleavage, with the subsequent dissociation of a 5kDa peptide (i.e. 44 a.a.) and a 95kDa membrane protein. The human NTR3 receptor/sortilin contains 831 amino acid residues and possesses a long cysteine-rich extracellular domain, a single transmembrane region and a short cytoplasmic tail (Petersen et al., 1997; Mazella et al., 1998). This receptor belongs to a newly defined family of heterogenous type-1 receptors that are characterized by similar N-terminal domains related to the yeast vacuolar sorting protein, Vps10p, which acts as sorting receptor for molecules in the secretory pathway and on the cell membrane. This receptor family, in addition to sortilin, includes the sortilin-related receptor-containing LDLR class A repeats (SorLA) and sortilin-related Vps10 domain-containing receptor (SorCS) -1, -2 and -3. The Vps10p domain in sortilin, which can be considered the archetypal member, makes up the entire extracellular region, whereas additional unrelated ectodomains are found in the other four receptors (Jacobsen et al., 1996; Yamazaki et al., 1996; Petersen et al., 1997; Hermey et al., 1999; Kikuno et al., 1999; Nagase et al., 2000; Mazella, 2001). All these receptors are widely expressed in embryonic and adult neural tissues and are considered to be involved in a number of functions, including endocytosis of ligands, targeting proteins in the biosynthetic pathway for Golgi-endosome transport and transmembrane signaling following activation of the receptor (Hermans-Borgmeyer et al., 1999; Mazella, 2001; Sarret et al., 2003; Hermey et al., 2004; Chen et al., 2005).

2.4. Distribution of sortilin

Lin and colleagues (1997) used northern blot analysis to demonstrate that sortilin is highly expressed in the CNS and lungs, with lower levels in the heart and muscle, and virtually absent from the liver. In the adult rat brain, sortilin mRNA and protein have been shown to be widely distributed in various regions, including the cortex, striatum, hippocampus, cerebellum and brainstem regions (Petersen et al., 1997; Lin et al., 1997; Sarret et al., 2003). At the cellular level, the majority of the receptor (~90%) is believed to reside in intracellular compartments, in particular, the Golgi apparatus (Petersen et al., 1997; Mazella, 2001; Navarro et al., 2001; Nielsen et al., 2001). Using confocal laser microscopy, it has also been demonstrated that sortilin is localized primarily in the trans-Golgi region of COS-1 cells transfected transiently with full-length sortilin cDNA (Petersen et al., 1997). This is corroborated by further studies on the C13NJ human microglial cell line where sortilin is found to be localized in the syntaxin-6-positive trans-Golgi compartment (Martin et al., 2003). Under certain conditions, sortilin is translocated from intracellular compartments to the cell membrane. Using immunoadsorption of Glut4-containing vesicles (Lin et al., 1997) and cell surface biotinylation of membranes (Kandror and Pilch, 1994), it has been shown that insulin can cause translocation of sortilin to the cell surface.

2.5. Ligands of sortilin

Several studies have been undertaken to elucidate the ligands that can interact with sortilin. One issue that is becoming increasingly clear is the unrelated nature of these ligands. This receptor, apart from neurotensin, can also bind other ligands, including receptor-associated protein (RAP), sphingolipid activator protein (SAP), lipoprotein lipase (LpL), and the 44-amino acid peptide cleaved during maturation of the receptor precursor (Petersen et al., 1997; Willnow, 1998; Nielsen et al., 1999; Munck-Petersen et al., 1999; Lefrancois et al., 2003). Nonetheless, very little is currently known regarding the role of endogenous sortilin in mediating central and/or peripheral effects of neurotensin or other ligands.

2.6. Physiological role of sortilin

When transfected into epithelial cell lines, sortilin can bind and internalize neurotensin efficiently, which is followed by the recycling of the receptors to the cell membrane after dissociation of the ligand (Navarro et al., 2001). Moreover, neurotensin stimulates the growth of CHO cells stably transfected with sortilin, suggesting a possible signaling role for the receptor in regulating cell proliferation (Dal Farra et al., 2001). This is supported, in part, by the demonstration that sortilin mediates neurotensin-induced migration of human microglial cells *via* stimulation of both MAP kinase and phosphatidylinositol-3 kinase pathways (Martin et al., 2003). There is also evidence to suggest that sortilin is essential in the formation of Glut4 storage vesicles in 3T3-L1 adipocytes, and the acquisition of insulin responsiveness in adipocyte cells (Shi and Kandror, 2005). Sortilin is capable of transporting soluble lysosomal enzymes from the trans-Golgi apparatus to the lysosomes (Ni and Morales, 2006). This receptor contains an extracellular Vps10p domain and a cytoplasmic domain which is similar to the cation-independent mannose-6-phosphate receptor involved in the intracellular trafficking of newly synthesized lysosomal enzymes. Assimilated evidence suggests that sortilin can bind to the adaptor protein Golgi-associated, γ-adaptin homologous, ARF binding protein (GGA) and mediate the transport of soluble lysosomal enzymes, such as prosaposin and GM2 ganglioside activator protein (GM2AP), from Golgi apparatus to the lysosomes (Petersen et al., 1997; Takatsu et al., 2001; Ni and Morales, 2006; Ni et al., 2006). Lefrancois et al. (2005) used a dominant negative form of sortilin, lacking the GGA binding motif, as well as small interfering RNA interference (siRNAi) method to show that SAPs also require sortilin to be trafficked to the lysosomal compartment in COS7 cells. Additionally, it has been reported that sortilin acts as a key regulator of sorting newly synthesized BDNF to appropriate secretory pathways in neurosecretory and neuronal cells (Chen et al., 2005).

The role of sortilin in pro-inflammatory responses has been demonstrated by

Dicou et al. (2004) who showed that the receptor can mediate neurotensin-induced expression of various cytokines/chemokines such as interleukin 1β (IL-1β), macrophage inflammatory protein-2 (MIP2), monocyte chemotactic protein 1 (MCP-1), TNFα and interleukin-6 (IL-6) in the N11 murine microglial cell line. These results suggest that sortilin may play a role in the recruitment and/or activation of brain macrophages, resulting in pro-inflammatory process that may contribute to pathophysiology of cerebral degenerative disorders such as Parkinson's disease and AD (Martin et al, 2005).

3. Link between sortilin and $p75^{NTR}$

Recently that Hempstead and colleagues have shown that sortilin binds directly to pro-NGF and pro-BDNF *via* their respective pro-domains and forms a receptor complex with $p75^{NTR}$ at the cell surface. This sortilin-$p75^{NTR}$ receptor complex appears to be involved in transducing the apoptotic effects of pro-NGF and pro-BDNF in cultured superior cervical ganglion cells. On the other hand, blocking the interaction of sortilin with pro-NGF/pro-BDNF inhibits apoptosis, thus suggesting a potential role for sortilin in $p75^{NTR}$-mediated cell death in cultured neurons (Nykjaer et al., 2004; Teng et al., 2005). These studies postulate that sortilin acts as a co-receptor and molecular switch which allows neurons expressing $p75^{NTR}$ to respond to pro-neurotrophins and consequently trigger pro-apoptotic events. In the absence of sortilin, extracellular proteases may cleave pro-NGF to mature NGF, which then promotes Trk-mediated survival signals (Nykjaer et al, 2004). Recently, Volosin et al. (2006) have demonstrated that sortilin is involved in pro-NGF-mediated cell death in $p75^{NTR}$-positive basal forebrain neurons under *in vivo* paradigm. They have shown that kainic acid-induced seizures can elicit the production of pro-NGF in astrocytes prior to activation of caspases in $p75^{NTR}$-positive neurons located in the basal forebrain region, thus raising the possibility that pro-NGF may induce $p75^{NTR}$-mediated apoptotic signaling *in vivo*. This is substantiated by two lines of experimental evidence: i) kainic acid treatment induces a decreased loss of neurons in the basal forebrain region of $p75^{NTR}$-/- mice compared with wild-type animals, and ii)

lysates from the basal forebrain region of kainic acid-treated rats induce the loss of basal forebrain cultured neurons, which can be prevented by antibodies to pro-NGF, $p75^{NTR}$ and sortilin.

4. Hypothesis and objectives

On the basis of the aforementioned results, I hypothesize that **sortilin may be expressed in $p75^{NTR}$-containing neurons in the adult rat brain and its level/ expression may be altered following *in vivo* administration 192 IgG-saporin.**

To address this issue my specific objectives are:

1. to determine the distribution of sortilin in adult rat brain and its possible localization in neurons expressing $p75^{NTR}$ and/or cholinergic phenotype.

2. to determine possible alterations in sortilin expression/levels in selected brain regions following 192 IgG-saporin-induced degeneration of the $p75^{NTR}$-expressing neurons.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS AND METHODS

1. Materials

Adult male Sprague Dawley rats (250-300g) used in all experiments were obtained from Health Sciences Laboratory Animal Services, University of Alberta, Edmonton, Alberta and handled in accordance with the University of Alberta and Canadian Council on Animal Care Guidelines. Rabbit anti-sortilin antiserum was generously provided by C.M. Petersen (University of Aarhus, Denmark), goat anti-choline acetyltransferase (ChAT), rabbit anti-vesicular acetylcholine transporter (VAChT) and mouse anti-$p75^{NTR}$ anti-sera were from Chemicon Intl. (Temecula, CA), mouse anti-glial fibrillary acidic protein (GFAP) was from Sigma (Oakville, Canada), and mouse anti-ED1 was from Serotec (Raleigh, NC). Horseradish peroxidase-conjugated secondary antibodies and anti-β-actin antiserum were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Texas Red- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA), and the elite Vectastain ABC kit was from Vector Laboratories (Burlingame, CA). Polyacrylamide electrophoresis gels (4–20%) were purchased from Invitrogen (Burlington, Canada), whereas the enhanced chemiluminescence (ECL) kit was from New England Nuclear (Mississauga, Canada). The immunotoxin 192 IgG-saporin was purchased from Cedarlane (Hornby, Canada). All other chemicals were purchased from Fisher Scientific (Burlingham, Canada) or Sigma.

2. Methods

2.1. 192 IgG-saporin intracerebroventricular (icv) administration

Adult male rats were anesthetized by sodium pentobarbital (i.p., 65 mg/kg) and mounted on a stereotaxic frame. Each animal received a bilateral injection of either 192 IgG-saporin (0.4 μg/μl; 5 μl/ventricle) or an equivalent volume of phosphate-buffered saline (PBS) through a 26-gauge Hamilton syringe into the lateral ventricles at the following coordinates: AP -1.4 mm, ML +1.8 mm, DV -3.5 mm, relative to Bregma. Following each injection, the cannula was left in place for 3 min to allow for diffusion of the injected substrate. Animals were then

fixed by perfusion at 1, 4, 7, 14 and 28 days post-treatment (6-8 animals/group) for immunocytochemical staining or killed and selected brain regions [i.e., medial septum/DBB, hippocampus and striatum] were collected for Western blotting.

2.2. *Western blotting*

To determine the distribution of sortilin in the normal brain, six adult rats were decapitated and their brains were removed immediately. Areas of interest (i.e., olfactory bulb, frontal cortex, parietal cortex, striatum, hippocampus, brainstem and cerebellum) were dissected out and processed for Western blotting. Additionally, brain tissues from selected regions [i.e., medial septum/DBB, hippocampus and striatum] of control and 192 IgG-saporin-treated animals were processed, in parallel, for Western blotting as described earlier (Hawkes et al., 2005). Briefly, all tissues were first homogenized in RIPA-lysis buffer [20 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Igepal CA-630, 50 mM NaF, 1 mM $NaVO_3$, 10 μg/ml leupeptin and 10 μg/ml aprotinin] and then dissolved in reduced sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% [w/v] sodium dodecyl sulfate, 1% glycerol, and 0.1% bromophenol blue). Proteins were then separated by 4-20% polyacrylamide gel electrophoresis for 90 min before being transferred to Hybond-C Nitrocellulose membranes. Membranes were then blocked for 1 hr with 8% non-fat milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.2% Tween-20 (TBST) and incubated overnight at 4°C with rabbit anti-sortilin (1:5,000) or goat-anti-ChAT (1:500) antibodies. Membranes were washed three times with TBST, incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1 hr at room temperature and then visualized using an ECL detection kit (Hawkes and Kar, 2004). Blots were subsequently stripped and re-probed with anti-β-actin (1:1,000) to ensure equal protein loading. All blots were quantified using a Microcomputer Imaging Device (MCID) analysis system, and values obtained as relative optical densities (ROD). The ROD values for the various markers/ROD actin, represented as a percentage of control, were plotted on bar graphs for the different days of 192 IgG-saporin post-treatment. The data, which are presented as mean ± SEM, were

analyzed using one-way analysis of variances (ANOVA) followed by Newman-Keuls *post-hoc* analysis with significance set at $p < 0.05$.

2.3. Immunohistochemistry

Adult rats from normal control (i.e., untreated rats) and experimental groups (i.e. 192 IgG-saporin- and saline-treated rats) were fixed by transcardial perfusion and their brains were processed for immunohistochemical staining. Briefly, the rats were anesthetized with 8% chloral hydrate (VWR Canlab, Montreal, Canada) and then perfused transcardially first using 0.01M PBS (pH 7.2), followed by 4% paraformaldehyde. The brains were removed and post-fixed overnight in the same fixative, and then stored in 30% PBS-sucrose at 4°C. Brains were sectioned coronally (20 or 40 μm) using a cryostat, collected in anti-freeze solution and then processed for immunohistochemical staining using the free-floating procedure as described earlier (Hawkes et al., 2006).

a) Enzyme-linked immunohistochemistry

For the enzyme-linked immunohistochemical procedures, 40 μm sections were washed in PBS, treated with 1% hydrogen peroxide for 30 min and then incubated overnight at room temperature with rabbit anti-sortilin (1:1,000), mouse anti-$p75^{NTR}$ (1:1,000), and goat-anti-ChAT (1:1,000) antibodies. Sections were then washed with PBS, exposed to avidin-biotin reagents for 1 hr, and then developed with the glucose-oxidase-diaminobenzidine tetrahydrochloride (DAB)-nickel enhancement method, as described previously (Jafferali et al., 2000). Briefly, sections were washed twice in 0.1M acetate buffer [pH 6.0 adjusted with 10% acetic acid], followed by 10-20 min incubation with glucose oxidase-DAB-nickel solution. This was followed by two rinses with 0.1M acetate buffer and two rinses with PBS. Sections were mounted on slides, air dried and subsequently dehydrated through graded alcohols prior to mounting in Permount mounting media. Immunostained sections were then examined and photographed using a Zeiss Axioskop-2 microscope.

b) Double immunofluorescence labeling

For double immunofluorescence staining, 20 μm brain sections from control and different experimental groups were incubated overnight with a combination of goat anti-ChAT (1:250), rabbit anti-sortilin (1:300), mouse anti-$p75^{NTR}$ (1:200), mouse anti-GFAP (1:1000) or mouse anti-ED1 (1:50), rinsed with PBS and then exposed to the appropriate Texas Red- and FITC-conjugated secondary antibodies (1:200) for 2 hr at room temperature. The sections were then washed thoroughly with PBS, cover-slipped with Vectashield mounting medium (Vector Laboratories) and visualized under a Zeiss Axioskop-2 fluorescence microscope. Photomicrographs were then processed using Adobe photoshop 6.0.

CHAPTER 3

RESULTS

RESULTS

1. The normal adult rat brain

1.1. Sortilin in the normal adult rat brain

Immunoblot analysis was performed to characterize antiserum's ability to recognize the native sortilin in the adult rat brain. As shown in Figure 3.1, the antiserum recognized a single band with an apparent molecular weight of 95 kDa, corresponding to the sortilin. As evident from a representative immunoblot, sortilin was present at all major regions of the brain, including olfactory bulb, frontal cortex, parietal cortex, striatum, hippocampus, brainstem and cerebellum. The overall expression of sortilin was found to be relatively lower in the olfactory bulb and striatum compared to other brain regions. At the cellular level, sortilin immunoreactivity is widely distributed, primarily in neuronal cell bodies and fibers, throughout the brain. The following section entails the distribution of immunoreactive sortilin observed in specific brain regions.

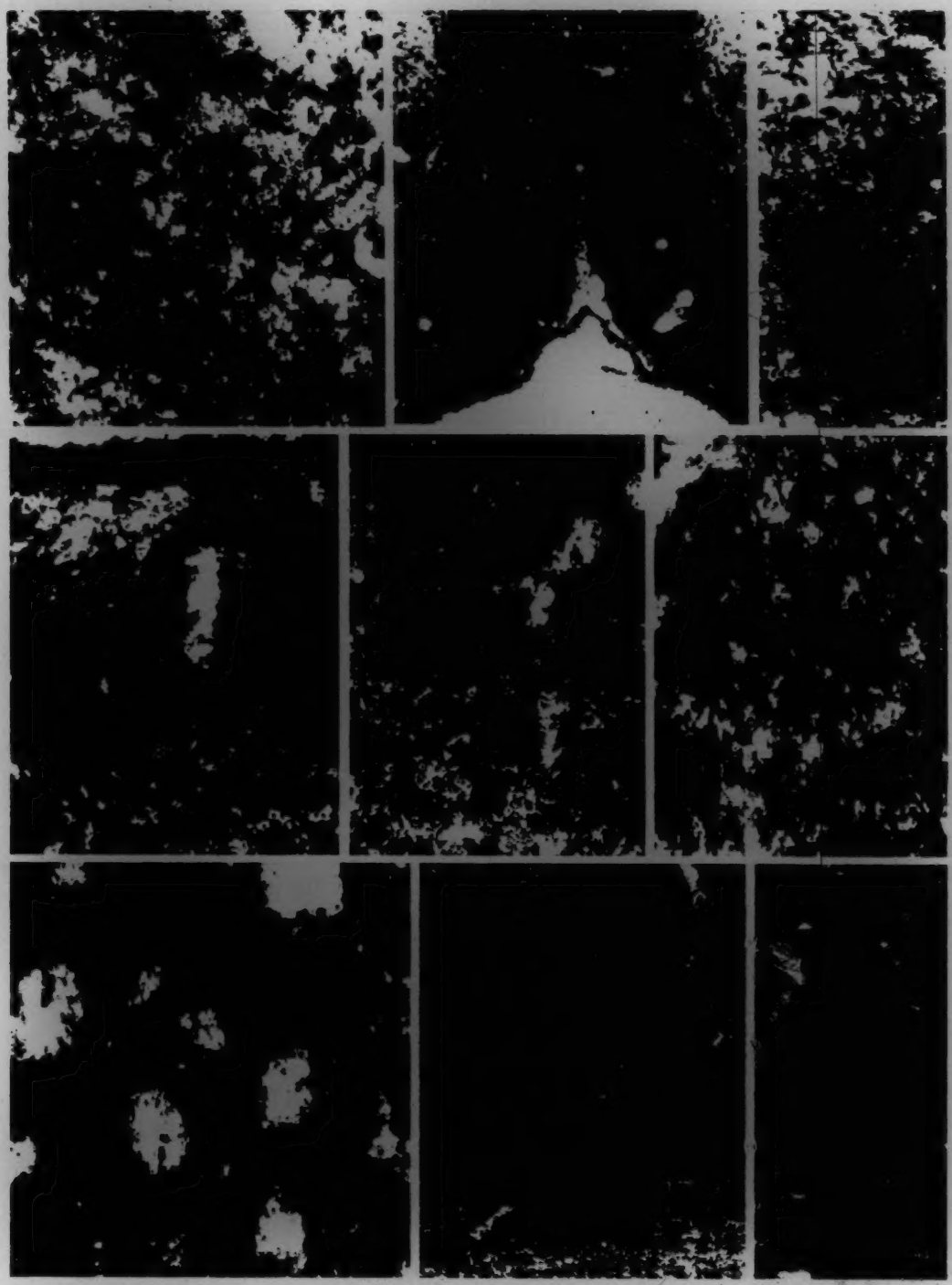
Basal forebrain and basal ganglia: Sortilin immunoreactivity was observed in all subfields of the basal forebrain including the septum, DBB and NBM (Figure 3.2A-E). In septal nuclei, a group of multipolar cells were moderately labeled (Figure 3.2A-C), whereas in the diagonal band complex, some weakly labeled neurons were found intermingled with moderately labeled neurons (Figure 3.2D). A number of sortilin-immunoreactive neurons were also seen in the bed nucleus of the stria terminalis and NBM (Figure 3.2E). Additionally, intensely labeled sortilin immunoreactive neurons were found scattered throughout the globus pallidus (Figure 3.2F) and striatum (Figure 3.2G).

Cerebral cortex: Sortilin immunoreactivity was detected in most layers of the neocortex with varying degrees of staining intensity. The labeling was high in layers IV-VI, moderate in layers II-III and relatively low in layer I (Figure 3.2H, I). The laminar distribution of sortilin-labeled neurons was particularly striking in the cingulate cortex and in the frontoparietal cortex. In the piriform cortex, intensely labeled sortilin immunoreactive neurons were evident along with a

Figure 3.1 Western blot of sortilin in different regions of the adult rat brain. Sortilin antiserum recognized a single band of approximately 95kDa, corresponding to the native sortilin protein. Amount of protein loading has been shown by re-probing the blot with anti-actin (lower panel) antiserum. OB, olfactory bulb; C, frontal cortex; P, parietal cortex; ST, striatum; H, hippocampus; BS, brainstem; CB, cerebellum.

Sortilin
Actin
OB
C
P
ST
H
BS
CB

Figure 3.2 Photomicrographs of transverse sections of the adult rat brain showing the distribution of sortilin immunoreactive neurons and fibres in the lateral septum (A), medial septum (B, C), diagonal band of Broca (D), nucleus basalis of Meynert (E), globus pallidus (F), striatum (G) and frontal cortex (H, I). Note that the labeling of sortilin is confined mostly to the cell soma and, to some extent, to the processes.



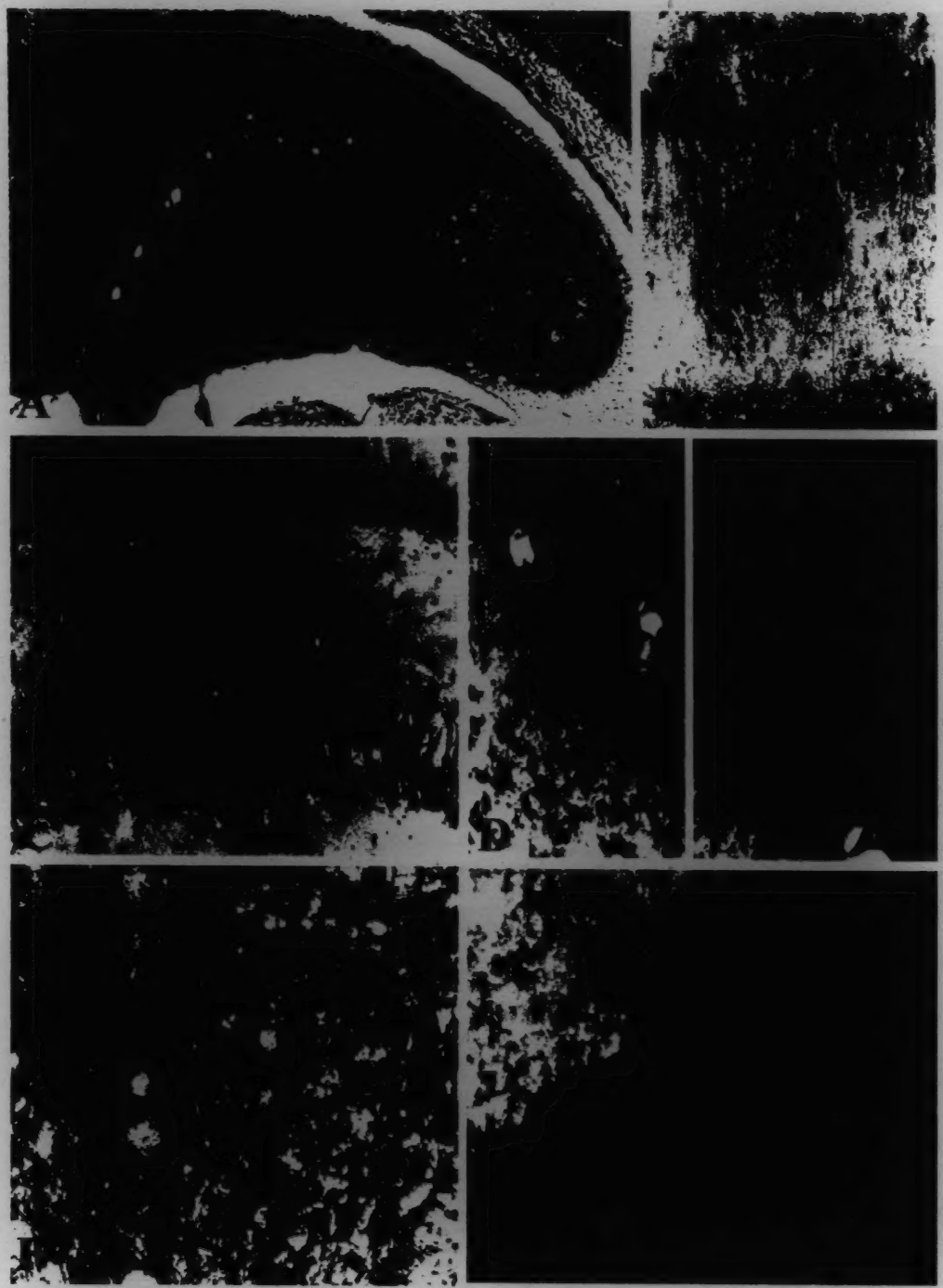
smaller population of weakly labeled neurons.

Hippocampus and Amygdala: The hippocampal formation showed some of the most intense sortilin immunoreactivity in the brain (Figure 3.3A-C). Within the Ammon's horn, strong labeling was apparent in the CA1-CA3 pyramidal cells and their apical dendrites (Figure 3.3A, B), but occasional multipolar neurons were also found scattered in the strata oriens and stratum radiatum. Within the dentate gyrus, granule cell somata were moderately labeled, whereas little sortilin immunoreactivity was evident in the adjacent molecular layer. A number of sortilin-positive polymorphic neurons were also observed in the hilus region of the hippocampus (Figure 3.3C). In the amygdaloid complex, several groups of moderately labeled sortilin-immunoreactive cell bodies were evident in the cortical, medial and basolateral amygdaloid nuclei. Additionally, some multipolar cells exhibiting rather weak immunoreactivity were also apparent in the anterior amygdaloid area and central amygdaloid nucleus.

Hypothalamus and Thalamus: In the hypothalamus rather moderate neuronal labeling was observed in the supraoptic and paraventricular nuclei, lateral hypothalamic area, lateral preoptic area, arcuate nucleus and mammillary bodies, whereas neurons located in the anterior hypothalamus showed rather weak labeling. A number of medium-sized sortilin immunostained neurons were observed throughout the thalamus (Figure 3.3D, E). These neurons which were moderately labeled were evident in anterior dorsal, anterior ventral, reticular, medial thalamic nuclei and the ventral and posterior nuclear groups.

Midbrain: Moderate somatodendritic labeling was observed in the superficial gray layers of the superior colliculus and in the central gray matter. The substantia nigra pars reticulata was characterized by multipolar neurons with moderate sortilin immunoreactivity, whereas the pars compacta exhibited rather weak labeling. Neurons of the red and oculomotor nuclei also displayed moderate immunoreactivity. Weakly labeled neurons were apparent in the ventral tegmental

Figure 3.3 Photomicrographs of transverse sections of adult rat brain showing the distribution of sortilin immunoreactive neurons and fibres in the hippocampus (A), pyramidal cell layers of CA1 region of the hippocampus (B), the granule cell layer and hilus of dentate gyrus (C), thalamus (D-lower magnification, E-higher magnification), motorneurons of the brainstem (F) and the Purkinje cells of the cerebellum (G). Note the intense sortilin labeling in the hippocampal neurons of the adult rat brain.


A
B

area, intermediate gray layer of superior colliculus and mesencephalic trigeminal nucleus.

Brainstem: Sortilin immunoreactivity was visible at all brainstem levels. A population of large multipolar neurons was encountered in the pontine reticular nucleus, whereas numerous moderately labeled neurons were seen in the inferior colliculus, abducens nucleus and reticulotegmental nucleus of pons. However, relatively strong labeling was evident particularly in the motor trigeminal nucleus, pontine nucleus and in the facial as well as vestibular nuclei (Figure 3.3F).

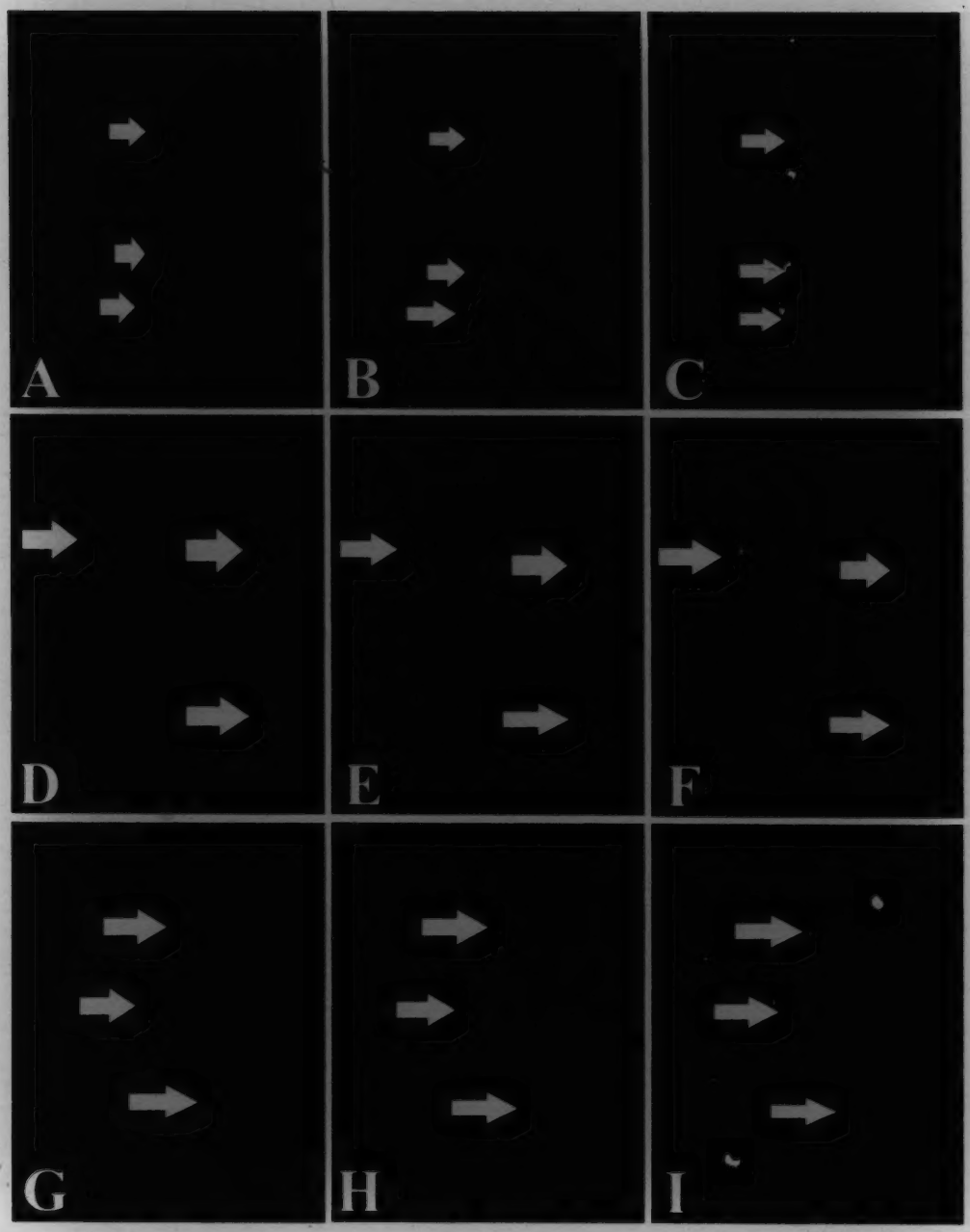
Cerebellum: A common pattern of sortilin immunoreactivity prevailed throughout the cerebellum. In the cortex, Purkinje cells were rather intensely stained and often seen in continuity with their stained dendritic shafts extending into the molecular layer (Figure 3.3G). The granule cells exhibited rather weak staining, whereas a number of deep cerebellar nuclei showed numerous moderately labeled immunoreactive cell bodies.

1.2. $p75^{NTR}$ and ChAT in the normal adult rat brain

Earlier studies have shown that majority of the basal forebrain cholinergic neurons express $p75^{NTR}$ receptor (Torres et al., 1994; Rossner, 1997). In keeping with these results, my double labeling experiments revealed that all ChAT-positive cholinergic neurons located in the medial septum (Figure 3.4A-C) and in the vertical and horizontal limbs of the diagonal band complex (Figure 3.4D-F) express $p75^{NTR}$ immunoreactivity. In the NBM, the majority of the cholinergic neurons express $p75^{NTR}$, but some ChAT-positive neurons were found to be $p75^{NTR}$ negative (Figure 3.4G-I). Additionally, striatal interneurons and motorneurons of the brainstem which exhibit ChAT immunoreactivity were found not to express $p75^{NTR}$.

1.3. Sortilin and ChAT in the normal adult rat brain

To determine if sortilin is localized in basal forebrain cholinergic neurons, I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performed double immunofluorescence labeling experiments using antisera against ChAT and sortilin. My dual labeling studies revealed that sortilin is co-localized with all ChAT-positive cholinergic neurons in the medial septum (Figure 3.5A-F), DBB (Figure 3.5G-L) and NBM (Figure 3.6A-F). In addition, the cholinergic interneurons of the striatum (Figure 3.6G-I) and motorneurons of the brainstem region (Figure 3.6M-O) exhibited sortilin immunoreactivity.

1.4. Sortilin and p75^{NTR} in the normal adult rat brain

After establishing that majority of the basal forebrain cholinergic neurons exhibit p75^{NTR} immunoreactivity, I wanted to evaluate the possible expression of sortilin in p75^{NTR} positive neurons. My dual labeling experiments clearly revealed that immunoreactive sortilin is localized in all p75^{NTR}-positive neurons in the medial septum (Figure 3.7A-F), vertical and horizontal limbs of the diagonal band complex (Figure 3.7G-L) and NBM (Figure 3.8A-F). While many sortilin-positive neurons located throughout the brain, including the brainstem motorneurons, were negative for p75^{NTR} immunoreactivity (Figure 3.8G-I), Purkinje cells of the cerebellum expressed both p75^{NTR} and sortilin immunoreactivity (Figure 3.8J-L).

2. 192 IgG-saporin-treated rat brain

2.1. Cholinergic system in 192 IgG-saporin-treated rat brain

To determine whether degeneration of basal forebrain cholinergic neurons expressing p75^{NTR} can lead to a concomitant alteration of sortilin levels, I first evaluated the time-dependent effects of 192 IgG-saporin administration on cholinergic neurons located in the basal forebrain region of the adult rat brain. A single icv injection of 192 IgG-saporin, as reported earlier (Torres et al., 1994; Rossner, 1997; Wiley, 2001; Hawkes et al., 2005), induced an extensive bilateral loss of ChAT-immunoreactive cell bodies in the basal forebrain areas [i.e., septum, vertical and horizontal limbs of DBB and NBM] from day 4 post-injection onwards (Figure 3.9A, D). At the cellular level, some sections displayed a few residual ChAT-positive neurons in the NBM, but not in other areas of the

Figure 3.5 Double immunofluorescence photomicrographs of transverse sections of control adult rat brain showing the distribution of sortilin (A, D, G, J) and ChAT (B, E, H, K) immunoreactivities and their co-localization (C, F, I, L) in the medial septum (A-F) and diagonal band of Broca (G-L). As evident from the figures, all ChAT-positive cholinergic neurons are found to express sortilin. Figures D-F and J-L are higher magnification of Figures A-C and G-I, respectively. Solid arrows indicate neurons exhibiting co-localization of sortilin and ChAT, whereas open arrows show a lack of co-localization between sortilin and ChAT.

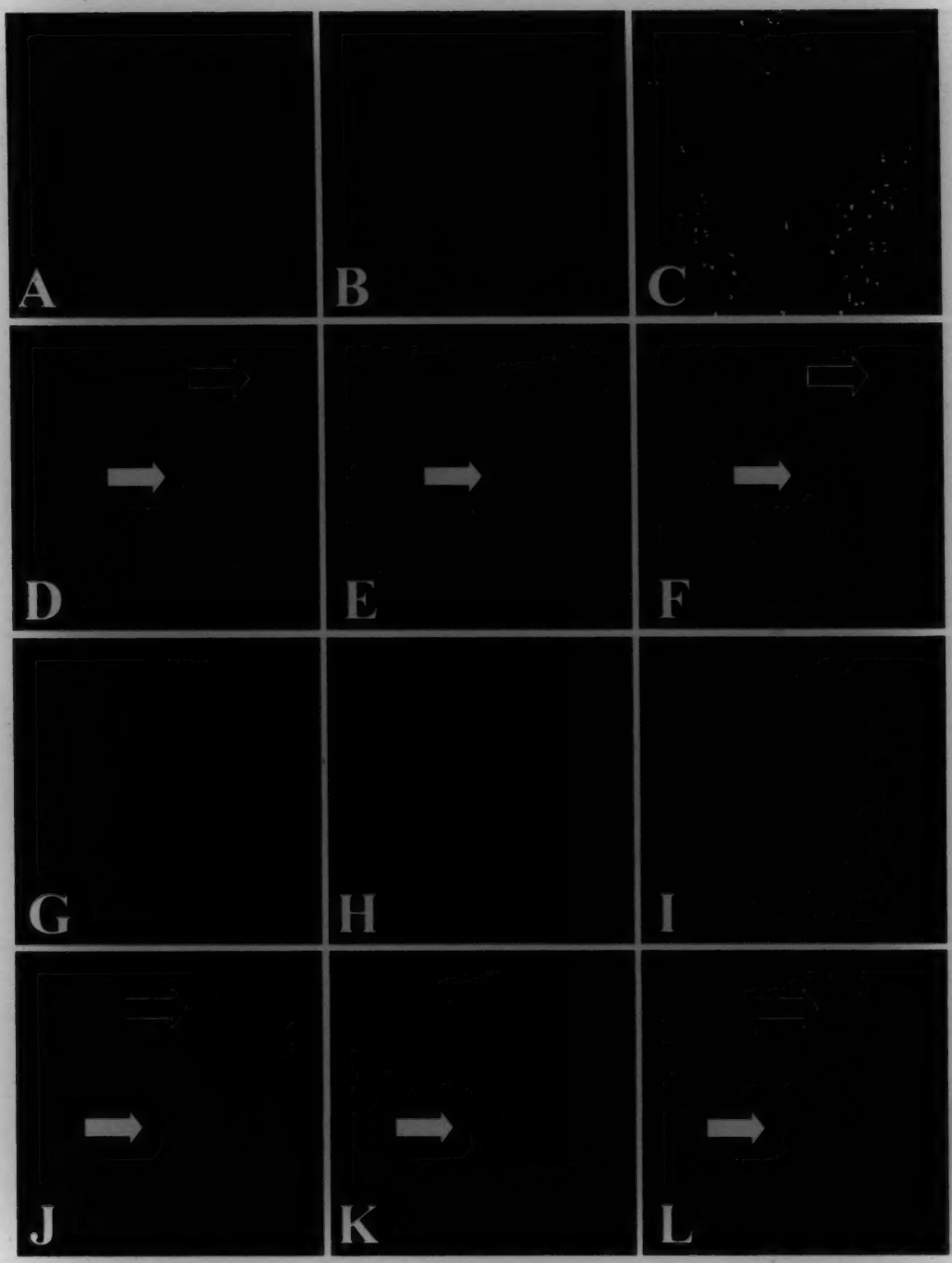
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Figure 3.6 Double immunofluorescence photomicrographs of transverse sections of control adult rat brain showing the distribution of sortilin (A, D, G, J, M) and ChAT (B, E, H, K, N) immunoreactivities and their possible co-localization (C, F, I, L, O) in the nucleus basalis of Meynert (A-F), striatum (G-I), hippocampus (J-L) and brainstem motorneurons (M-O). A subset of sortilin-positive neurons in the nucleus basalis shows ChAT immunoreactivity (A-F). Co-localization of sortilin and ChAT is also seen in cholinergic interneurons of the striatum, pyramidal layer fibres of the hippocampus, as well as in motorneurons of the brainstem. Figures D-F are higher magnifications of Figures A-C. Solid arrows indicate neurons exhibiting co-localization of sortilin and ChAT, whereas open arrows show a lack of co-localization between sortilin and ChAT.

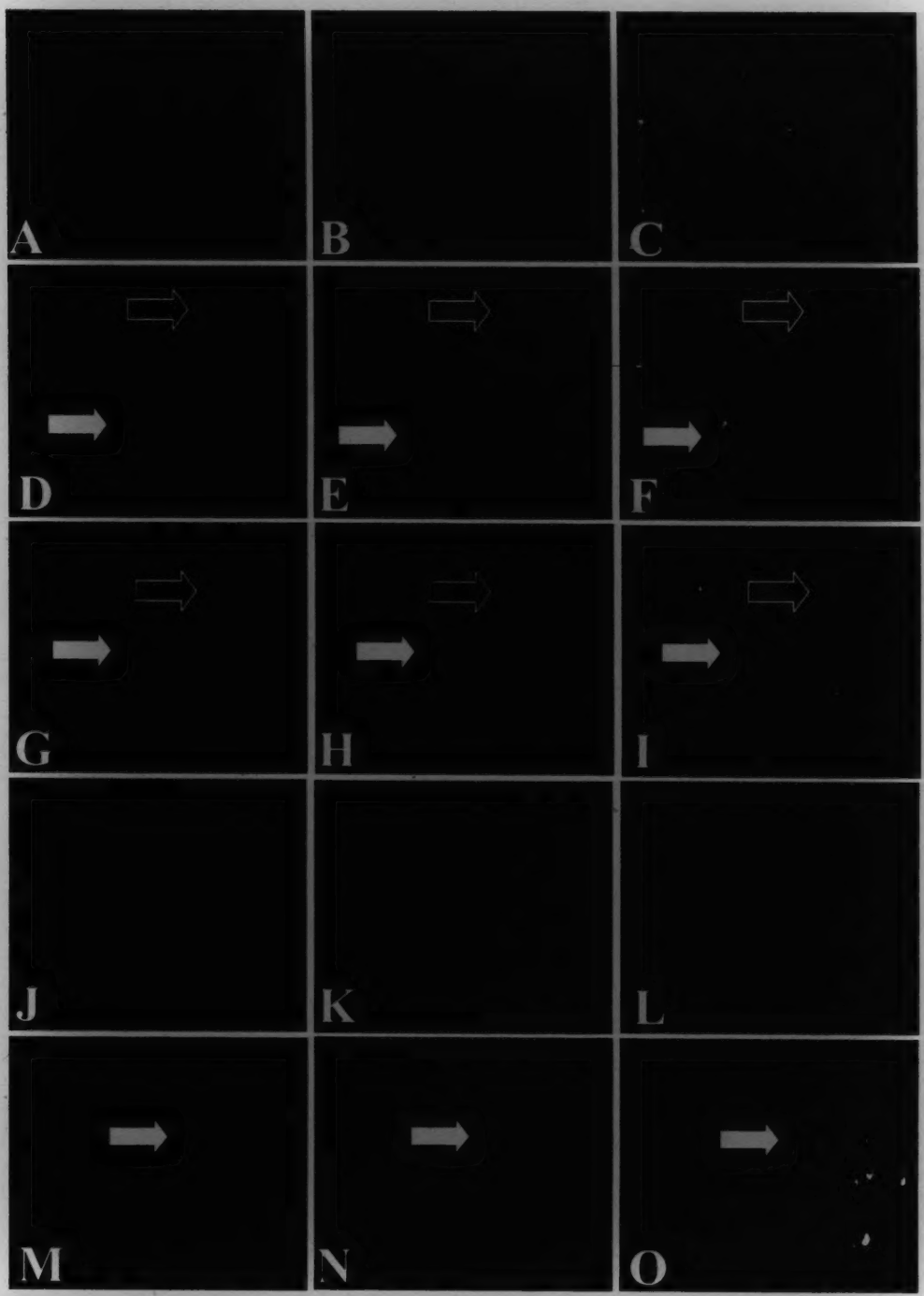
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Figure 3.7 Double immunofluorescence photomicrographs of transverse sections of control adult rat brain showing the distribution of sortilin (A, D, G, J) and $p75^{NTR}$ (B, E, H, K) immunoreactivities and their possible co-localization (C, F, I, L) in the medial septum (A-F) and diagonal band of Broca (G-L). As evident from the figures, a subset of sortilin-positive neurons in these areas exhibit $p75^{NTR}$-immunoreactivity. Figures D-F and J-L are higher magnifications of Figures A-C and G-I, respectively. Solid arrows indicate neurons exhibiting co-localization of sortilin and $p75^{NTR}$, whereas open arrows show a lack of co-localization between sortilin and $p75^{NTR}$.

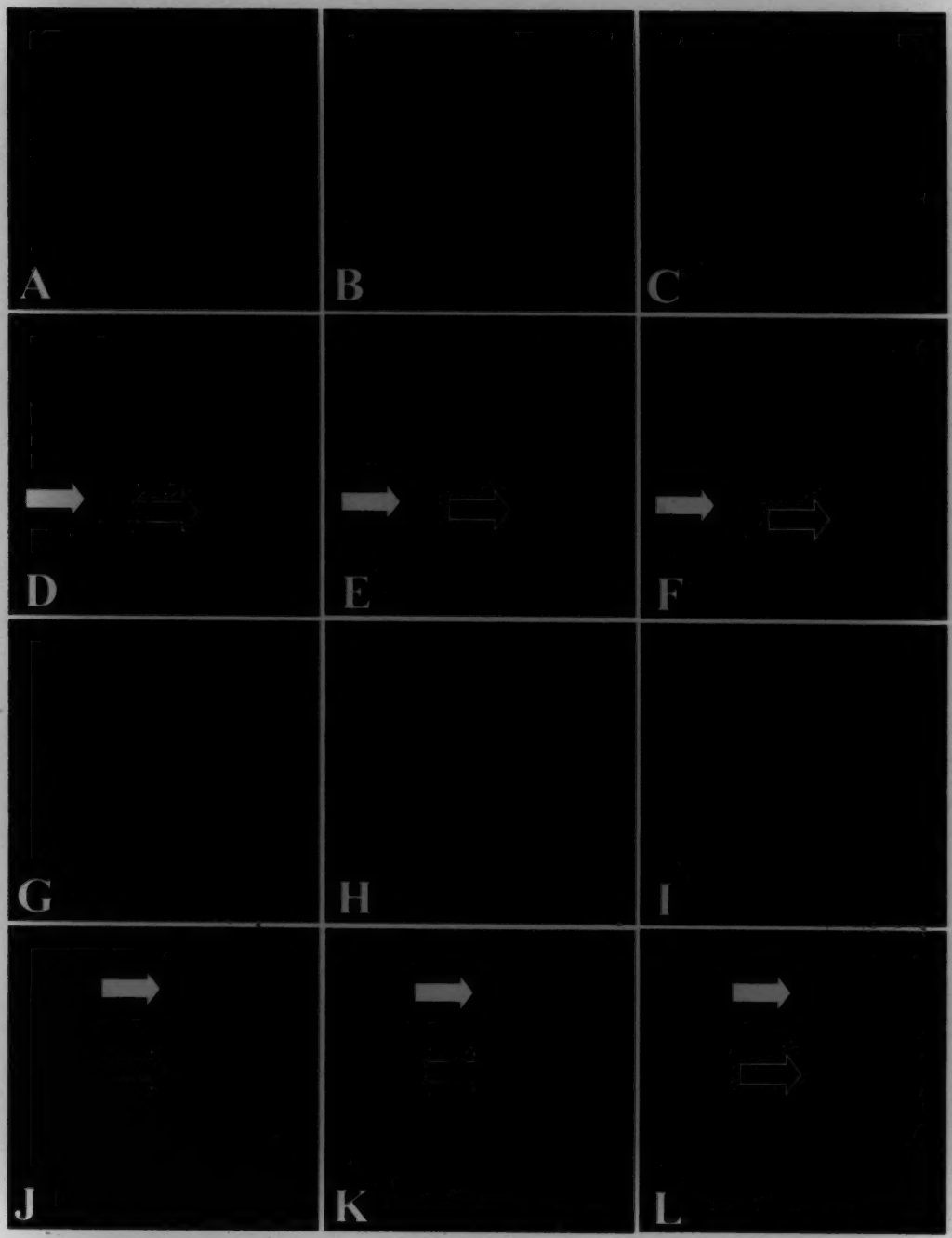
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Figure 3.8 Double immunofluorescence photomicrographs of transverse sections of control adult rat brain showing the distribution of sortilin (A, D, G, J) and $p75^{NTR}$ (B, E, H, K) immunoreactivities and their possible co-localization (C, F, I, L) in the nucleus basalis of Meynert (A-F), motorneurons of the brainstem (G-I) and Purkinje cells of the cerebellum (J-L). Note that sortilin and $p75^{NTR}$ are co-localized in the nucleus basalis (A-F) and cerebellum (J-L), but not in the brainstem motorneurons (G-I). Figures D-F are higher magnifications of Figures A-C. Solid arrows indicate neurons exhibiting co-localization of sortilin and $p75^{NTR}$, whereas open arrows show a lack of co-localization between sortilin and $p75^{NTR}$.

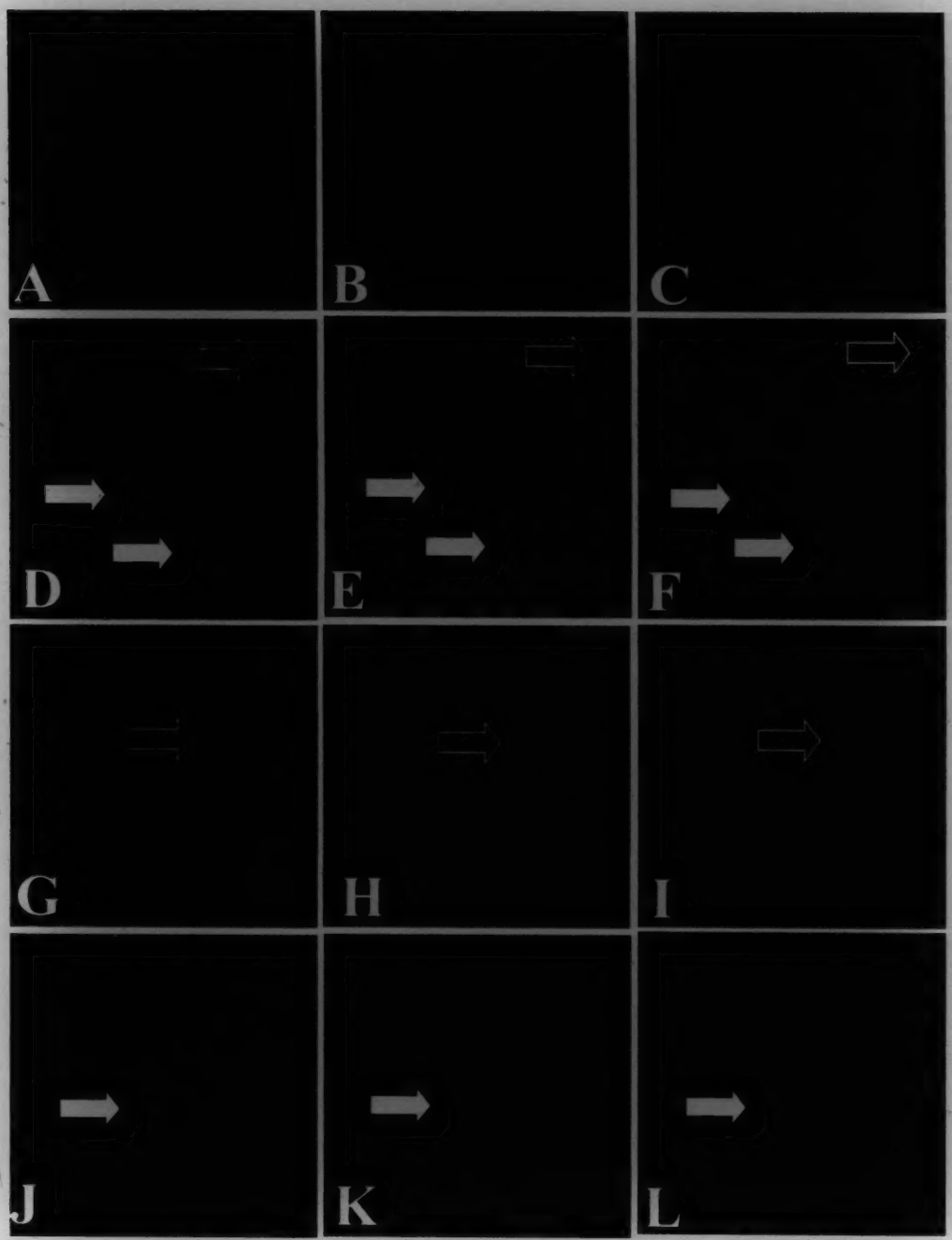
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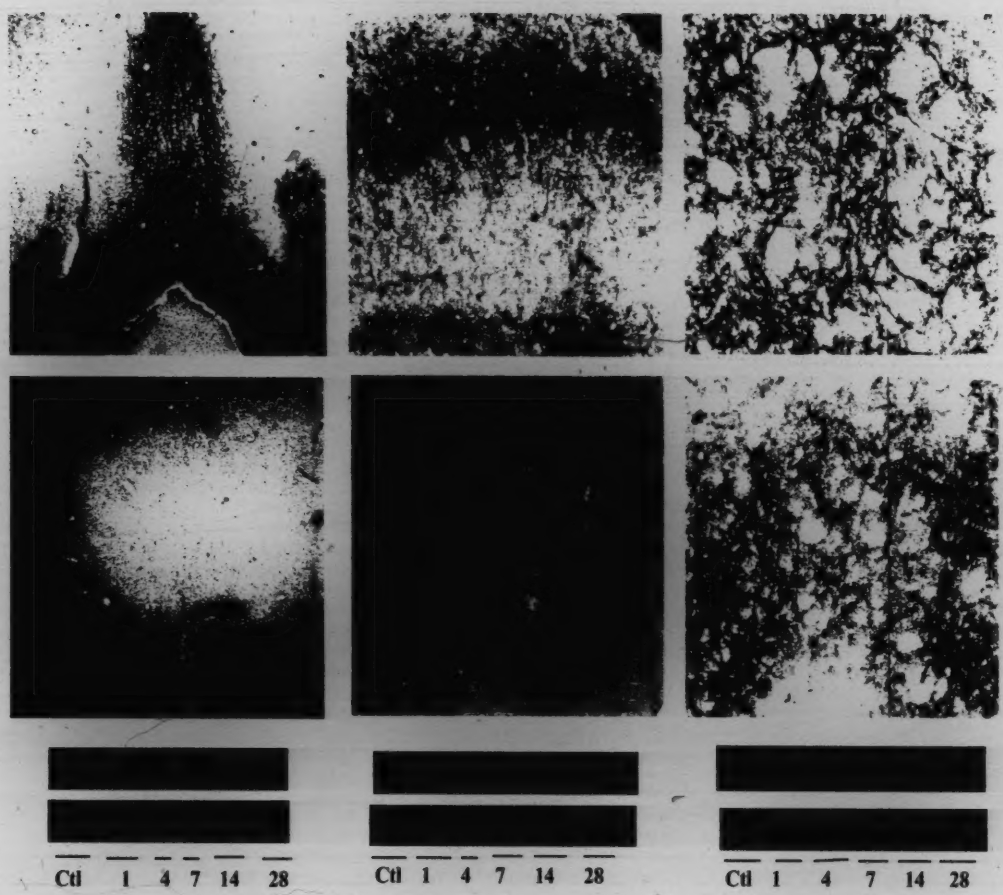
Figure 3.9 Photomicrographs showing the distribution of ChAT immunoreactivity in the medial septum/diagonal band of Broca (A, D), hippocampus (B, E) and striatum (C, F) of control animals (A-C) and in animals 7 days after treatment with 192 IgG-saporin (D-F). Bilateral injection of 192 IgG-saporin induced an almost complete loss of cholinergic neurons by day 7 after treatment in the medial septum/diagonal band of Broca (A, D) and their fibre projections to the hippocampus (B, E), whereas cholinergic interneurons within the striatum remained unaffected (C, F). G-L; Western blots and histograms depicting alterations in ChAT levels at 1, 4, 7, 14, 28 days in the septum/diagonal band of Broca (G, J), hippocampus (H, K) and striatum (I, L) following administration of 192 IgG-saporin relative to levels in saline-treated controls (Ctl). Histograms (J-L) represent mean ± SEM ChAT levels from at least three separate experiments, each of which was replicated 3-4 times. Note the significant decrease in ChAT levels from 4 day post-treatment onwards in the septum/diagonal band of Broca and hippocampus but not in the striatum of 192 IgG-saporin-treated animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (ANOVA followed by Neuman-Keuls *post hoc* test).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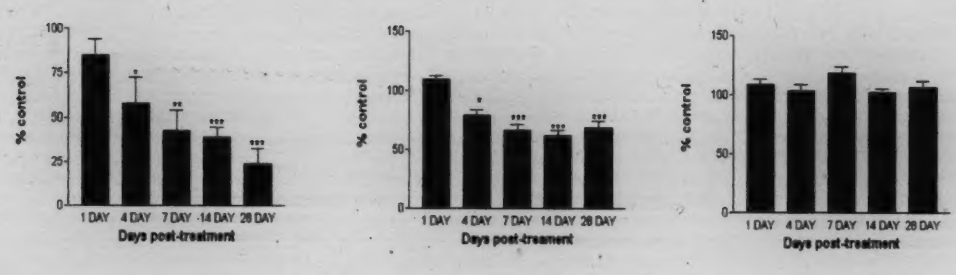
Ctl 1 4 7 14 28
Ctl 1 4 7 14 28

G H I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Days post-treatment

J K L

medial septum and DBB. As expected, the loss of cholinergic cell bodies was accompanied by a parallel loss of ChAT-positive fibers in the hippocampus (Figure 3.9B, E). The cholinergic interneurons of the striatum, in keeping with earlier studies (Heckers et al., 1994; Rossner, 1997), were unaffected by 192 IgG-saporin treatment (Figure 3.9C, F). These morphological data were substantiated by Western blot analysis which showed a statistically significant reduction in ChAT enzyme levels in the medial septum/DBB and hippocampus, but not in the striatum, at 4, 7, 14 and 28 days following administration of 192 IgG-saporin. One day-treated rats did not show any significant alteration in ChAT levels in any regions of the brain (Figure 3.9G-L).

2.2. $p75^{NTR}$-positive neurons in 192 IgG-saporin treated rat brain

In keeping with the loss of cholinergic neurons, $p75^{NTR}$ immunoreactivity was found to be completely absent in medial septum, vertical and horizontal limbs of DBB and NBM region of 192 IgG-saporin treated rats from 7-day post-treatment onwards (Figure 3.10A, C). A corresponding loss of $p75^{NTR}$-positive fibers was observed in the hippocampus of the treated rats (Figure 3.10B, D). Earlier studies have shown that $p75^{NTR}$ immunoreactivity is usually not present in the striatum of the normal adult rat brain but is induced following metabolic or excitotoxic injury (Kokaia et al., 1998; Andsberg et al., 2001; Greferath et al., 2002; Hanbury et al., 2002). In contrast to these results, $p75^{NTR}$ expression was not evident at any time in the striatal region of the 192 IgG-saporin-treated rats.

2.3. Sortilin in 192 IgG-saporin treated rat brain

Given the evidence that $p75^{NTR}$ is co-expressed with sortilin, I evaluated the levels/ expression of sortilin at different times in selected brain regions following administration of 192 IgG-saporin (Figure 3.11A-L). My Western blot analysis revealed that sortilin levels are not significantly altered at any time (i.e., 1, 4, 7, 14 and 28 days post lesion) either in the septum/DBB complex, hippocampus or striatal region of the immunotoxin-treated rats compared to saline-treated control

Figure 3.10 Photomicrographs showing the distribution of $p75^{NTR}$ immunoreactivity in the medial septum/diagonal band of Broca (A, C), hippocampus (B, D) of control animals (A, B) and in animals 7 days post-treatment with 192 IgG-saporin (C, D). Bilateral injection of 192 IgG-saporin induced an almost complete loss of $p75^{NTR}$-positive neurons by day 7 post-treatment in the medial septum/diagonal band of Broca (A, C) and their fibre projections to the hippocampus (B, D).

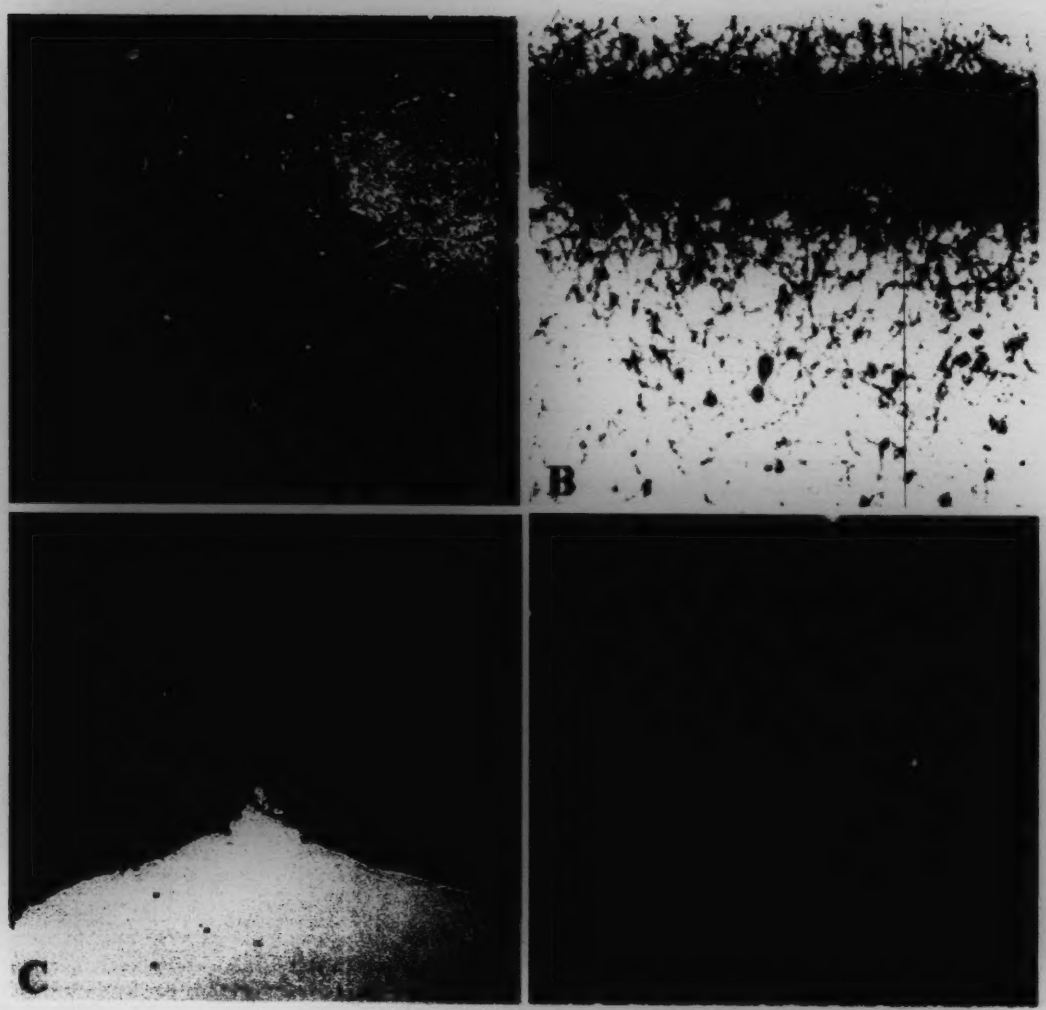
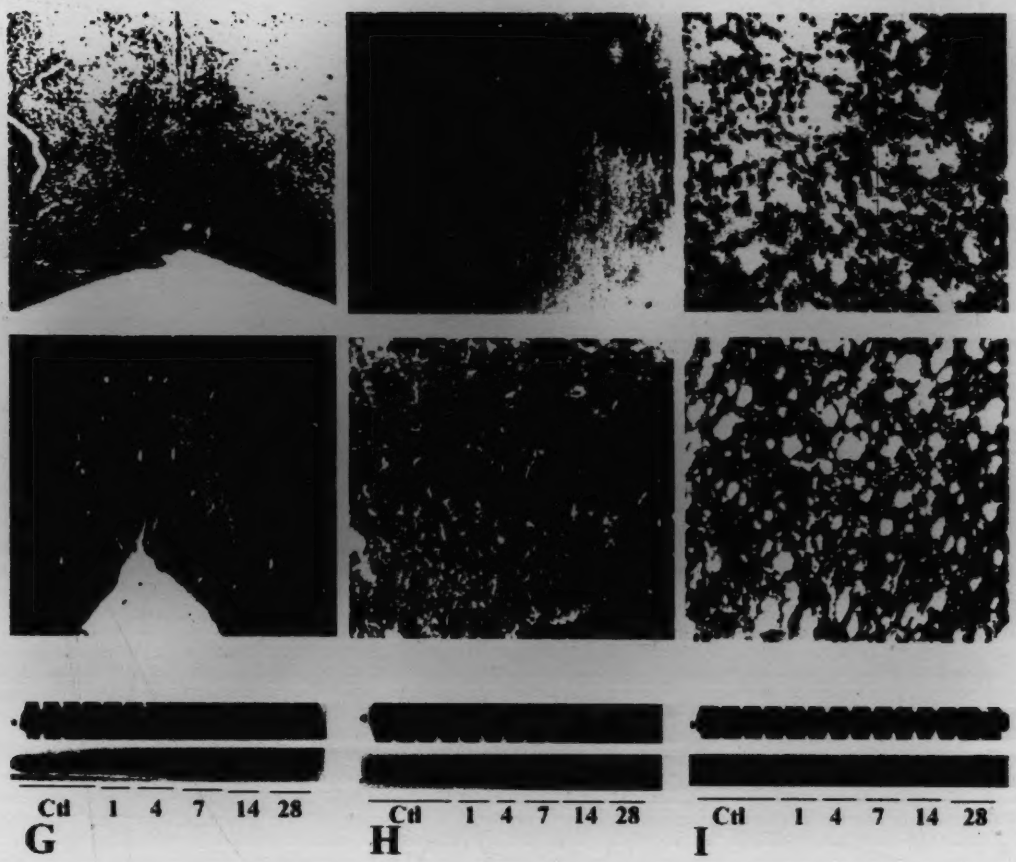

B
C

Figure 3.11 Photomicrographs showing the distribution of sortilin immunoreactivity in the medial septum/diagonal band of Broca (A, D), hippocampus (B, E) and striatum (C, F) of control animals (A-C) and in animals 7 days after treatment with 192 IgG-saporin (D-F). Bilateral injection of 192 IgG-saporin did not induce visible alteration in sortilin immunoreactivity either in the medial septum/diagonal band of Broca (A, D), hippocampus (B, E) or striatum (C, F). Western blots and histograms showing no significant change in sortilin levels in the septum/diagonal band of Broca (G, J), hippocampus (H, K) and striatum (I, L) following administration of 192 IgG-saporin relative to saline-treated controls (Ctl). Histograms (J-L) represent mean ± SEM sortilin levels from at least three separate experiments, each of which was replicated 3-4 times. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (ANOVA followed by Neuman-Keuls *post hoc* test).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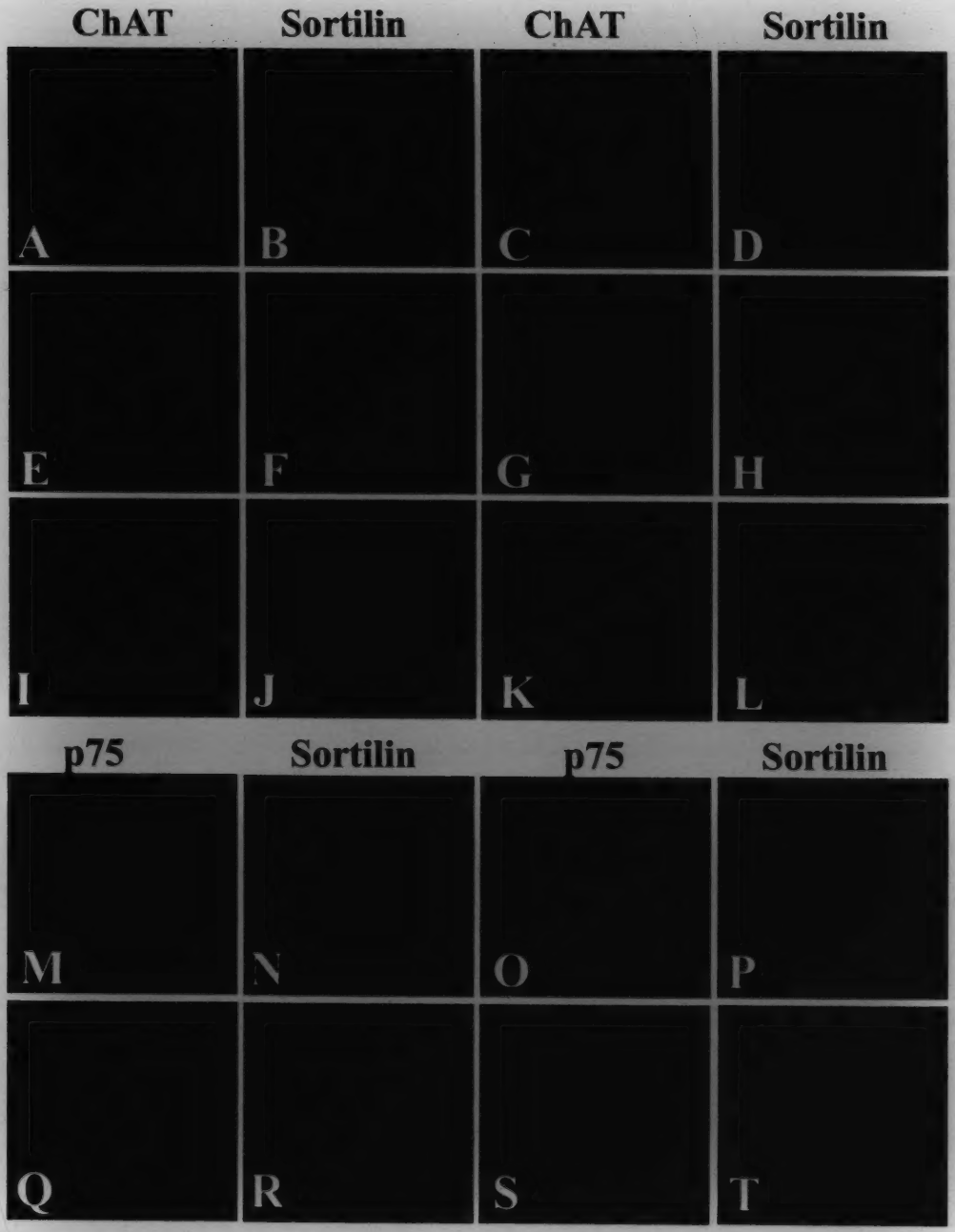
rats (Figure 3.11G-L). This is substantiated by my immunocytochemical data which showed no marked alteration in sortilin immunoreactivity in the basal forebrain or hippocampal regions following administration of 192 IgG-saporin (Figure 3.11A, B, D, E). Additionally, the cholinergic interneurons of the striatum which are $p75^{NTR}$-negative also showed no change in immunoreactive sortilin in the treated rats (Figure 3.11C, F). My dual labeling studies with ChAT/sortilin (see Figure 3.12A-L) and $p75^{NTR}$/sortilin (see Figure 3.12M-T) further revealed that selective degeneration of the $p75^{NTR}$-positive cholinergic neurons did not induce any observable change in sortilin immunoreactivity in surviving neurons that are located in the medial septum, DBB or NBM regions of the immunotoxin-treated rats.

Earlier studies have reported that selective loss of basal forebrain cholinergic neurons following administration of 192 IgG-saporin can induce activation of astrocytes and microglia in the basal forebrain regions (Hollerbach et al., 1998; Lemke et al., 1999). To determine whether immunotoxin treatment can trigger sortilin expression in glial cells, I performed double labeling experiments with sortilin and GFAP as well as sortilin and ED1 in the basal forebrain regions of 7day post-lesion rats (see Figure 3.13). My results clearly revealed that 192 IgG-saporin, as reported earlier (Hollerbach et al., 1998; Lemke et al., 1999), can induce activation of astrocytes as well as microglia in the basal forebrain regions of the treated rats. However, sortilin is not found to be expressed in GFAP-labeled astrocytes in the basal forebrain regions of either control (Figure 3.13B, F) or 192-IgG saporin-treated (Figure 3.13D, H) rats. Similarly, ED1-labeled reactive microglia, which are usually not apparent in control rats (Figure 3.13J, N), did not exhibit sortilin immunoreactivity in immunotoxin-treated rats (Figure 3.13L, P).

Figure 3.12 Double immunofluorescence photomicrographs of transverse sections of the adult rat brain showing the distribution of ChAT (A, C, E, G, I, K) and sortilin (B, D, F, H, J, L) immunoreactivities in the diagonal band of Broca (A-D), nucleus basalis of Meynert (E-H) and striatum (I-L) of control rats (A, B, E, F, I, J) and of rats 7 days after treatment with 192 IgG-saporin (C, D, G, H, K, L). There is a loss of ChAT immunoreactivity in the DBB (A, C) and nucleus basalis (E, G) 7 days after saporin treatment. However, striatal cholinergic interneurons are resistant to saporin toxicity (I, K). There is also no visible change in sortilin expression in the diagonal band of Broca (B, D), nucleus basalis (F, H) and striatum (J, L) following saporin treatment.

Double immunofluorescence photomicrographs of transverse sections of the adult rat brain showing the distribution of $p75^{NTR}$ (M, O, Q, S) and sortilin (N, P, R, T) immunoreactivities in the diagonal band of Broca (M-P) and nucleus basalis of Meynert (Q-T) of control (M, N, Q, R) and 7 day 192 IgG-saporin-treated rats (O, P, S, T). As evident from the photomicrographs, saporin administration induced a complete loss of $p75^{NTR}$ immunoreactivity in the diagonal band of Broca (M, O) and nucleus basalis of Meynert (Q, S), but no visible alteration in sortilin expression in the respective regions (N, P, R, T) of the treated rats.


ChAT
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p75
Sortilin
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Figure 3.13 Double immunofluorescence photomicrographs of transverse sections of the adult rat brain showing the distribution of immunoreactive sortilin (A, C, E, G) and GFAP (B, D, F, H) in the medial septum (A-D) and diagonal band of Broca (E-H) of control rats (A, B, E, F) and in rats after 7 days treatment with 192 IgG-saporin (C, D, G, H). Note that sortilin is not expressed in GFAP-labeled astrocytes either in control or in saporin-treated rats.

Double immunofluorescence photomicrographs of transverse sections of the adult rat brain showing the distribution of sortilin (I, K, M, O) and ED1 (J, L, N, P) in the medial septum (I-L) and diagonal band of Broca (M-P) of control rats (I, J, M, N) and after 7 days treatment with 192 IgG-saporin (K, L, O, P). Note that sortilin immunoreactivity is not evident in ED1-labeled microglia either in control or in saporin-treated rats.

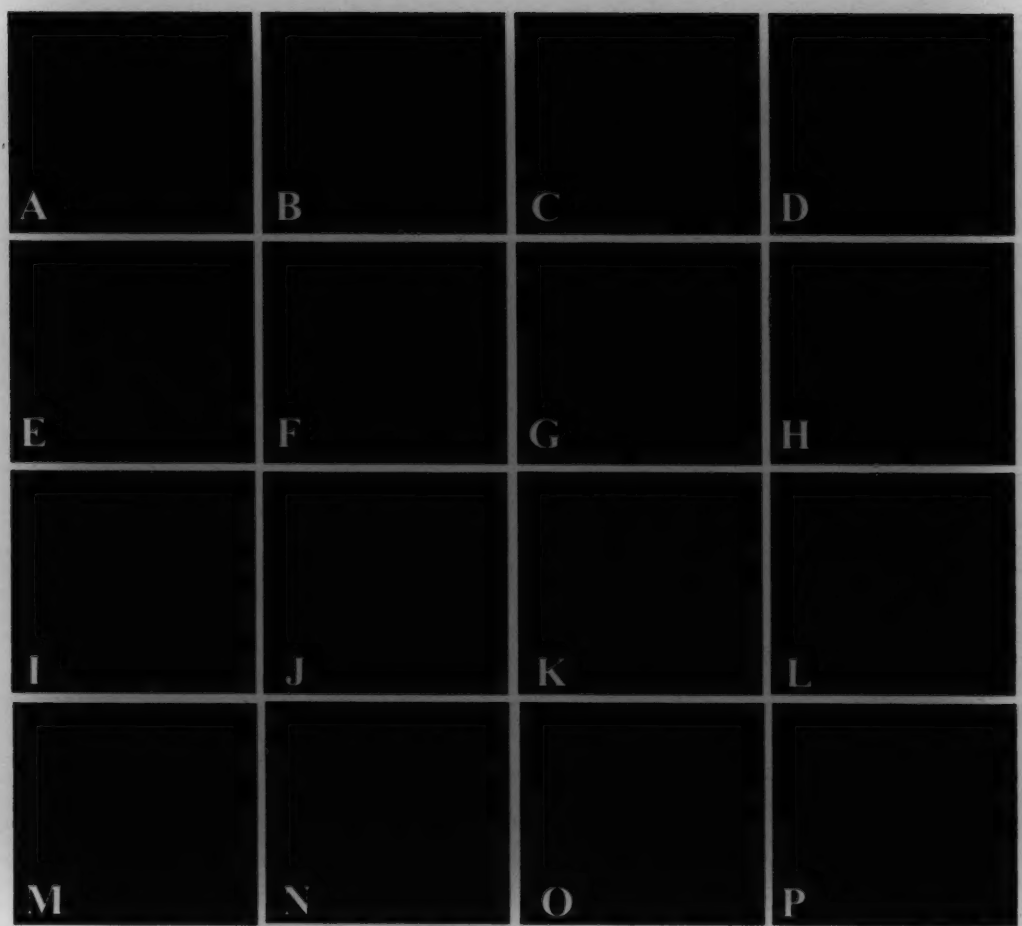
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Table 3.1. Distribution and co-localization of sortilin with ChAT-positive as well as $p75^{NTR}$ -positive neuronal cell bodies in the adult rat brain. The number of neuronal cell bodies observed in different brain regions are depicted as high (+++), moderate (++), low (+) and absent (-).

Area	Sortilin	ChAT	$p75^{NTR}$
Cortex			
Layer I	-	-	-
Layer II	+++	-	-
Layer III	+++	-	-
Layer IV	+++	-	-
Layer V	+++	-	-
Basal forebrain			
Medial septum	+++	++	++
Lateral septum	+++	-	-
Diagonal band of Broca	+++	++	++
Nucleus basalis of Meynert	+++	++	++
Basal ganglia			
Nucleus accumbens	++	-	-
Caudate-putamen	+++	++	-
Globus pallidus	++	-	-
Claustrum	++	-	-
Amygdala			
Central nucleus	++	-	-
Basomedial nucleus	+	-	-
Basolateral nucleus	++	-	-
Hippocampus			
CA1	+++	+	+
CA2	+++	+	+
CA3	+++	+	+
Dentate gyrus			
Granule cells	++	-	-
Hilus	++	-	-
Thalamus			
Anterior dorsal nucleus	+++	-	-
Anterior ventral nucleus	+++	-	-
Medial thalamic nuclei	++	-	-
Medial geniculate nucleus	++	-	-
Lateral geniculate nucleus	+++	-	-
Hypothalamus			
Supraoptic nucleus	+++	-	-
Paraventricular nucleus	++	-	-
Arcuate nucleus	+++	-	-
Mammillary nucleus	++	-	-
Brainstem			
Superior colliculus	++	-	-
Inferior colliculus	++	-	-
Red nucleus	++	-	-
Periaqueductal grey	+	-	-
Motor trigeminal nucleus	++	++	-
Cerebellum			
Cerebellar cortex			
Granule cell layer	+	-	-
Purkinje cell layer	++	-	++

CHAPTER 4

DISCUSSION

DISCUSSION

My results demonstrate that sortilin is widely distributed in all regions of the adult rat brain. A subset of sortilin-immunoreactive neurons located in the basal forebrain region and cerebellum was found to express $p75^{NTR}$ immunoreactivity. Additionally, sortilin immunoreactivity was evident in ChAT-positive cholinergic neurons of the basal forebrain, striatum and brainstem regions. Administration of 192 IgG-saporin induced a significant loss of ChAT and $p75^{NTR}$ immunoreactivity in the basal forebrain (i.e., medial septum, DBB and NBM) and hippocampal regions but not in the striatum. Interestingly, sortilin level/expression was not found to be significantly altered either in the basal forebrain or hippocampus of the immunotoxin-treated rats compared to saline-treated control rats. Collectively, these results provide evidence to suggest that although sortilin is co-localized with $p75^{NTR}$-positive cells, its levels/expression may be differentially regulated than $p75^{NTR}$ levels/expression.

1. Widespread distribution of sortilin in the normal adult rat brain

The results obtained in this study revealed that sortilin immunoreactivity, as reported earlier (Sarret et al., 2003), is widely distributed throughout the normal adult rat brain. At the cellular level, most of the immunoreactivity appears to be associated with the somatodendritic compartments but the intensity of staining varies distinctly from region to region within the brain. Areas that express relatively high levels of sortilin immunoreactivity include the striatum, deeper layers (layers IV and V) of the cortex, pyramidal and granule cell layers of the hippocampus, selected hypothalamic and thalamic nuclei, Purkinje cells of the cerebellum and motorneurons of the brainstem. Moderate to weak neuronal labeling was apparent in various brain regions, including basal forebrain areas, midbrain areas, superior colliculus and granule cells of the cerebellum. The distribution of sortilin-immunoreactive cell bodies also largely conformed to the localization of sortilin mRNA except in a few areas such as the medial geniculate nucleus and the dorsal raphe nucleus in which there were neurons weakly positive for sortilin mRNA but no immunoreactive cells (Sarret et al., 2003). This

discrepancy between mRNA expression and immunoreactivity may be due to variations in the stability of the messenger among different brain regions or to differential regulation of translation levels of the protein.

Many of the brain regions enriched with sortilin-immunoreactive neurons have previously been reported as being richly innervated by neurotensinergic axon terminals (Jennes et al., 1982; Emson et al., 1985), thereby supporting the concept that sortilin may act to regulate the function of neurotensin. These regions include the frontal cortex, the bed nucleus of the stria terminalis, the medial septum, amygdaloid nuclei, caudate putamen, various thalamic and hypothalamic nuclei and the spinal trigeminal nucleus (Jennes et al., 1982; Emson et al., 1985). It is of interest to note that only a small proportion of sortilin was found in association with the plasma membrane by electron microscopy in brain neurons (Sarret et al., 2003) and by subcellular fractionation in non-neuronal cells (Petersen et al., 1997; Morris et al., 1998), thus raising doubt about the ability of the receptor to regulate neuromodulation. However, this finding does not preclude the possibility that sortilin, as demonstrated for other receptor types, can be targeted to the plasma membrane under specific physiological and/or pharmacological conditions (Stroh et al., 2000; Cahill et al., 2001). Interestingly, sortilin immunoreactivity is also evident in many brain regions displaying neurotensin-positive cell bodies such as lateral septum, DBB, bed nucleus of stria terminalis, amygdaloid nuclei, substantia nigra, ventral tegmental area and periaqueductal gray (Jennes et al., 1982; Emson et al., 1985). Given the evidence that sortilin is associated mostly with the Golgi apparatus and is involved in sorting function (Morris et al., 1998; Nielsen et al., 1999, 2001), it is possible that sortilin localized within neurotensin-expressing cells may have a critical role in the intracellular sorting of neurotensin to vesicles.

The expression of sortilin is found to overlap extensively with that of the other two neurotensin receptors, NTR1 and NTR2 (Moyse et al., 1987; Boudin et al., 1996; Alexander and Leeman, 1998; Sarret and Beaudet, 2003). A number of

earlier studies have shown that exogenously applied neurotensin can be internalized into neurons in a NTR1-dependent manner in both *ex vivo* and *in vivo* paradigms (Castel et al., 1992; Faure et al., 1995). Given the established role of sortilin in the sorting of ligands to the trans-Golgi network (Morris et al., 1998; Nielsen et al., 1999, 2001), it is tempting to speculate that it may be involved in the internalization of neurotensin into neurons in which it is co-expressed with NTR1. Additionally, it is possible that sortilin can mediate the effects of neurotensin by forming a heterodimer with NTR1 or NTR2 as reported for several other neuropeptide receptors (Angers et al., 2002; Brady and Limbird, 2002). This is partly supported by experimental evidence which showed that sortilin and NTR1 can form complexes in HT29 intestinal cells that exhibit pharmacological properties different from those of NTR1 alone, specifically regarding the modulation of inositol phosphate turnover and MAP kinase activity (Martin et al., 2002). Thus, given the widespread distribution of sortilin over a variety of neuronal populations in adult rat brain, it is not unlikely to consider that the receptor may be involved, apart from its intracellular sorting/trafficking role, in the regulation of a wide spectrum of neurotensin functions such as hypothermia, pain, dopamine transmission and neuroendocrine effects (Nemeroff et al., 1992; Rosténe and Alexander, 1997; Sarret and Beaudet, 2003).

2. Expression of sortilin in $p75^{NTR}$-positive cholinergic neurons of the adult rat brain

Earlier studies have shown that ChAT-positive cholinergic neurons are widely but selectively distributed throughout the CNS (Semba and Fibiger, 1989; Everitt and Robbins, 1997; Kar, 2002; Mufson et al., 2003). A subset of central cholinergic neurons located in the basal forebrain region, which rely on NGF for their survival and maintenance, are known to express $p75^{NTR}$ (Hefti et al., 1985; Fischer et al., 1987; Kromer, 1987; Friedman et al., 1993). Although I and others have shown immunohistochemically that cholinergic interneurons of the adult rat striatum are $p75^{NTR}$-negative (Heckers et al., 1994; Rossner, 1997; Perry et al., 2001; Hawkes et al., 2005), Smith and colleagues (2004), using reverse

transcription-polymerase chain reaction (RT-PCR) have demonstrated the presence of $p75^{NTR}$ mRNA in these cells, thus suggesting that either low amounts and/or limited sensitivity of the immunohistochemical methods may preclude $p75^{NTR}$ detection in adult rat striatum. Cerebellar Purkinje cells, which are ChAT-negative are found to express $p75^{NTR}$ immunoreactivity, as reported earlier (Heckers et al., 1994; Waite et al., 1995; Perry et al., 2001). My double immunolabeling studies further revealed that sortilin is expressed in all ChAT-positive neurons located in the basal forebrain region (i.e., medial septum, DBB and NBM) and striatum as well as in brainstem motorneurons. Additionally, sortilin is found to be co-localized with $p75^{NTR}$ in both the basal forebrain and cerebellar Purkinje cells. These results, taken together, suggest that sortilin immunoreactivity is apparent in all ChAT- as well as $p75^{NTR}$-positive neurons in all major regions of the adult rat brain studied in the present investigation.

There is evidence that neurotensin can regulate endogenous acetylcholine release from the adult rat cortex (Lapchak et al., 1990) and can promote gamma and theta cortical activity (Cape et al., 2000). Although neurotensin receptors have been shown to be localized in the basal forebrain cholinergic neurons (Faure et al., 1995; Sarret and Beaudet, 2003), at present, the receptor subtype(s) involved in mediating the effects of neurotensin on central cholinergic system remains unclear. Given the evidence that sortilin is expressed in basal forebrain cholinergic neurons and can also regulate intracellular signaling (Martin et al., 2002, 2003), it will be of interest to determine the possible implication of this receptor in mediating the effects of neurotensin on cholinergic neurons. The presence of sortilin in all $p75^{NTR}$- positive basal forebrain cholinergic neurons is in agreement with a recent study which showed that expression and interaction between these two receptors may underlie Pro-NGF-mediated death of these neurons following kainic acid treatment (Volosin et al., 2006). Since $p75^{NTR}$ and sortilin can act as co-receptors in mediating apoptotic cell death in the basal forebrain region (Volosin et al., 2006), it would be of interest to evaluate whether all cells expressing both receptors are also TrkA-positive. This may help in

addressing whether the $p75^{NTR}$-sortilin-mediated "death signal" can be regulated or influenced by altered expression/levels of TrkA immunoreactivity.

3. Cholinergic system in 192 IgG-saporin-treated rat brain

The immunotoxin 192 IgG-saporin was constructed from 192 IgG, a monoclonal antibody to the rat low affinity NGF receptor and the ribosome-inactivating toxin saporin. This toxin is selectively taken up by neurons expressing $p75^{NTR}$ and retrogradely transported to the soma where it is cleaved, allowing saporin to inhibit ribosomal protein synthesis leading to neuronal death (Heckers et al., 1994; Rossner, 1997; Wiley, 2001). When administered directly into the cholinergic basal forebrain nuclei, 192 IgG-saporin is highly selective for the cholinergic basal forebrain neurons, but icv injection of the immunotoxin can affect all $p75^{NTR}$-expressing neurons in the brain (Torres et al., 1994; Rossner, 1997; Perry et al., 2001; Hawkes et al., 2005). This immunotoxin has been extensively used not only to evaluate the behavioral and neurochemical abnormalities observed following cholinergic hypofunction but also to model neuropathological consequences associated with AD pathology (Heckers et al., 1994; Rossner, 1997; Perry et al., 2001; Hawkes et al., 2005). In keeping with these results, I observed an almost complete degeneration of the forebrain cholinergic neurons by 7 days following a single icv administration of the immunotoxin. This was accompanied by a substantial depletion in the level/expression of ChAT in the hippocampus, which receives projections from basal forebrain cholinergic neurons (Semba and Fibiger, 1989; Everitt and Robbins, 1997). The striatal cholinergic interneurons, which do not express $p75^{NTR}$, were relatively spared. Additionally, a few $p75^{NTR}$-negative basal forebrain cholinergic neurons located in the ventral pallidum and ventral to the lenticular nucleus were found to be spared by the immunotoxin. This is consistent with earlier findings in rat (Heckers et al., 1994) and mouse (Berger-Sweeney et al., 2001) and is believed to be account for intact cholinergic innervations in the amygdala following 192 IgG-saporin administration. More recently, our lab has shown that 192 IgG-saporin-induced degeneration of basal forebrain cholinergic

neurons is accompanied by a decrease in the levels of phosphatidylinositol-3 kinase/phospho-Akt and increased glycogen synthase kinase-3β activity, thus raising the possibility that altered intracellular signaling, apart from ribosomal inactivation, may have a role in mediating the toxic effects of the immunotoxin (Hawkes et al., 2005).

4. Sortilin in 192 IgG-saporin-treated rat brain

Although numerous studies have shown that $p75^{NTR}$ can act as an apoptotic receptor during development and following injury, there has been controversy about the precise ligand requirements for these effects. Mature neurotrophins are not effective activators of $p75^{NTR}$-induced apoptosis as high non-physiological concentrations are often required to induce even modest levels of cell death (Barker, 2004; Bronfman and Fainzilber, 2004; Kaplan and Miller, 2004; Nykjaer et al., 2005). Using the furin-resistant form of pro-NGF, Hempstead and colleagues demonstrated for the first time that pro-NGF can bind $p75^{NTR}$ and can act as a potent inducer of $p75^{NTR}$-dependent apoptosis in sympathetic neurons, oligodendrocytes and in a vascular smooth muscle cell line (Lee et al., 2001). However, studies demonstrating that pro-NGF can bind equally well to $p75^{NTR}$ and TrkA but with ten-fold lower affinity than NGF raise questions about selectivity of the apoptotic role of $p75^{NTR}$ in cells expressing both types of these receptors (Nykjaer et al., 2004, 2005). The recent discovery that pro-NGF can act *via* a dual receptor system of $p75^{NTR}$ and sortilin to mediate cell apoptosis is considered to be a major breakthrough in establishing an apoptotic role for $p75^{NTR}$. Using multidisciplinary approaches, Nykjaer and colleagues have shown that pro-NGF interacts with both sortilin and $p75^{NTR}$ *via* its pro- and mature domains, respectively. However, the binding of pro-NGF to both receptors on the cell surface is necessary to generate high-affinity sites and to mediate apoptosis. Blocking the interaction of pro-NGF and sortilin, using a fusion protein formed by conjugation of the pro-domain of pro-NGF to glutathione S-transferase (GST), inhibits pro-NGF-mediated apoptosis, whereas normal expression of exogenous sortilin in Schwann cells, which usually express only $p75^{NTR}$, renders these cells

sensitive to the apoptotic effect of pro-NGF (Nykjaer et al., 2004). Additionally, it has also been demonstrated that pro-BDNF can induce apoptosis *via* interaction with the sortilin and $p75^{NTR}$ receptor complex in sympathetic neuronal cultures (Teng et al., 2005). However, it is of interest to note that co-expression of sortilin and $p75^{NTR}$ may not always lead to pro-neurotrophin-mediated cell death as pro-NGF treatment has been shown to induce migratory activity rather than death in melanoma cells expressing both sortilin and $p75^{NTR}$ (Shonukan et al., 2003; Barker, 2004).

Given the evidence that $p75^{NTR}$ is expressed in the basal forebrain cholinergic neurons whose effects are known to be regulated by neurotensin (Lapchak et al., 1990; Cape et al., 2000), I evaluated the possible co-expression of $p75^{NTR}$ and sortilin in these neurons of the adult rat brain. My immunohistochemical studies clearly demonstrated that both sortilin and $p75^{NTR}$ are evident in all the ChAT-positive basal forebrain neurons. This is supported by a recent study which showed the expression of both sortilin and $p75^{NTR}$ in cultured as well as adult rat brain basal forebrain neurons (Volosin et al., 2006). However, degeneration of these neurons by icv administration of 192 IgG-saporin did not induce any significant alteration in sortilin levels, as evident from my Western blot results, either in the basal forebrain region or in the hippocampus. To determine whether lack of alteration in the receptor level is due to compensatory adjustment from up-regulated levels in the surviving neurons or its induction in reactive glial cells, I subsequently evaluated the expression of the receptor in the affected regions of the treated rats using immunohistochemical methods. My results showed that the expression of sortilin is neither visibly up-regulated in surviving neurons nor it is induced in reactive astrocytes or microglia that are apparent following treatment with 192 IgG-saporin. Given the evidence that sortilin, unlike $p75^{NTR}$, is more widely expressed in various neuronal populations throughout the brain, it is possible that degeneration of a subset of cholinergic neurons induced only subtle changes in the level of this receptor which are not reflected in the Western blot analysis. Thus, further studies are needed to determine whether the

expression/level of sortilin mRNA is altered in the affected regions following treatment with 192 IgG-saporin. Also, it would be of interest to establish whether the levels of pro-NGF, as shown recently following kainic acid treatment (Volosin et al., 2006), are increased following treatment with 192 IgG-saporin.

5. Summary

In summary, the present study demonstrates that sortilin is expressed in $p75^{NTR}$- and ChAT-positive neurons in all major regions of the adult rat brain. The selective loss of $p75^{NTR}$-positive basal forebrain cholinergic neurons did not, however, induce a significant alteration either in the levels or expression of sortilin in the affected regions of the treated rats. These results, taken together, provide evidence to suggest that although sortilin is co-expressed with $p75^{NTR}$ positive cells, its levels/expression may not run in parallel with $p75^{NTR}$ levels or expression.

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